REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
15 Aug 14	Final	1 Jul 12- 30 Jun 14

4. TITLE AND SUBTITLE
Development of a Traumatic Brain Injury Assessment Score using Novel

5a. CONTRACT NUMBER
N/A

5b. GRANT NUMBER
HT9404-12-1-TS13

5c. PROGRAM ELEMENT NUMBER
N/A

6. AUTHOR(S)
Buonora, John E., PhD, RN, MAJ, AN, USA

5d. PROJECT NUMBER
N12-P12

5e. TASK NUMBER
N/A

5f. WORK UNIT NUMBER
N/A

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M. Jackson Fdn for the Adv. of Mil. Med. Inc.

6720-A Rockledge Drive, Suite 100, Bethesda, MD 20817

8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
TriService Nursing Research Program, 4301 Jones Bridge RD, Bethesda, MD 20814

10. SPONSOR/MONITOR'S ACRONYM(S)
TSNRP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
N12-P12

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
N/A

14. ABSTRACT

Purpose: There is no effective method to assess mild traumatic brain injury (mTBI) objectively. Brain-specific autoantibodies could be used to identify proteins that will serve as circulating biomarkers for the assessment of mTBI. **Design**: Prospective between subjects experimental design **Methods**: Autoimmune profiling was used to identify novel brain proteins targeted by TBI-induced autoantibodies to determine if these proteins contribute to a circulating biomarker signature useful in the diagnosis and assessment of mTBI. Immunosorbent electrochemiluminescent assays were developed for one of the discovered novel biomarker proteins (peroxiredoxin 6) and six established neuropathology biomarkers. **Sample**: Emergency room patient blood samples were obtained from two separate ongoing collaborating studies (Cohort 1: mild to moderate TBI; Cohort 2: moderate to severe TBI). Subjects were adults admitted with a diagnosis of head injury. Admission plasma samples were obtained from Cohort 1 (n = 154) and 2-7 days post-injury. Cohort 2 (n = 106) had plasma samples obtained at admission, 6, 12, and 24 hours post-injury. Both cohorts were compared to an individualized control group. **Analysis**: The study employed multivariate analysis of variance, utilizing seven dependent variables against one, two-level (control: injured) independent variable. **Findings**: Compared to controls the mean plasma values of 5 of the candidate TBI biomarker proteins in Cohort 1and Cohort 2 were significantly elevated at (admission and 2-7 days post-injury) and (6, and 12 hours post-injury) respectively. The summation of the fold-changes observed in the plasma levels of 5 biomarkers differentiated control samples from both the mild and the severe brain injured with scores of 5, 17, 32 respectively. **Implications for Military Nursing**: Creating a TBI assessment score for mTBI provides an opportunity to diagnose an endemic condition in our service members. Accurate and timely diagnosis of mTBI addresses a fundamental tenet guiding the scientific basis of nursing research, understanding and easement of the symptoms of acute illness.

15. SUBJECT TERMS

mild traumatic brain injury, biomarkers, mild and the severe brain injured, fit and ready force

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					Debra Esty
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	69	**19b. TELEPHONE NUMBER** *(include area code)*
unclassified	unclassified	unclassified			301-319-0596

TriService Nursing Research Program Final Report Cover Page

Sponsoring Institution	TriService Nursing Research Program
Address of Sponsoring Institution	4301 Jones Bridge Road Bethesda MD 20814
USU Grant Number	HT9404-12-1-TS13
USU Project Number	N12-P12
Title of Research Study or Evidence-Based Practice (EBP) Project	Development of a Traumatic Brain Injury Assessment Score using Novel Biomarkers Discovered Through Autoimmune Profiling
Period of Award	1 July 2012 – 30 June 2013; 1 July 2013 – 30 June 2014 no cost extension.
Applicant Organization	Henry M. Jackson Fdn for the Adv. of Mil. Med. Inc.,Uniformed Services University
Address of Applicant Organization	6720-A Rockledge Drive | Suite 100 | Bethesda, Maryland 20817

Principal Investigator (PI) Military Contact Information

Duty Title	[redacted]
Address	[redacted]
Telephone	[redacted]
Mobile Telephone	[redacted]
E-mail Address	[redacted]

PI Home Contact Information

Address	[redacted]
Telephone	[redacted]
Mobile Telephone	[redacted]
E-mail Address	[redacted]

Signatures

PI Signature _____ Date _____

Mentor Signature _____ Date _____

Table of Contents

Abstract

Purpose:
There is no effective method to assess mild traumatic brain injury (mTBI) objectively. Brain-specific autoantibodies could be used to identify proteins that will serve as circulating biomarkers for the assessment of mTBI.

Design:
Prospective between subjects experimental design

Methods:
Autoimmune profiling was used to identify novel brain proteins targeted by TBI-induced autoantibodies to determine if these proteins contribute to a circulating biomarker signature useful in the diagnosis and assessment of mTBI. Immunosorbent electrochemiluminescent assays were developed for one of the discovered novel biomarker proteins (peroxiredoxin 6) and six established neuropathology biomarkers.

Sample:
Emergency room patient blood samples were obtained from two separate ongoing collaborating studies (Cohort 1: mild to moderate TBI; Cohort 2: moderate to severe TBI). Subjects were adults admitted with a diagnosis of head injury. Admission plasma samples were obtained from Cohort 1 (n = 154) and 2-7 days post-injury. Cohort 2 (n = 106) had plasma samples obtained at admission, 6, 12, and 24 hours post-injury. Both cohorts were compared to an individualized control group.

Analysis:
The study employed multivariate analysis of variance, utilizing seven dependent variables against one, two-level (control: injured) independent variable.

Findings:
Compared to controls the mean plasma values of 5 of the candidate TBI biomarker proteins in Cohort 1and Cohort 2 were significantly elevated at (admission and 2-7 days post-injury) and (6, and 12 hours post-injury) respectively. The summation of the fold-changes observed in the plasma levels of 5 biomarkers differentiated control samples from both the mild and the severe brain injured with scores of 5, 17, 32 respectively.

Implications for Military Nursing:
Creating a TBI assessment score for mTBI provides an opportunity to diagnose an endemic condition in our service members. Accurate and timely diagnosis of mTBI addresses a fundamental tenet guiding the scientific basis of nursing research, understanding and easement of the symptoms of acute illness.

TSNRP Research Priorities that Study or Project Addresses

Primary Priority

Force Health Protection:	☒ Fit and ready force ☐ Deploy with and care for the warrior ☐ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☐

Secondary Priority

Force Health Protection:	☐ Fit and ready force ☐ Deploy with and care for the warrior ☐ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☐

Progress Towards Achievement of Specific Aims of the Study or Project - Findings related to each specific aim, research or study questions, and/or hypothesis:

The research proposal submitted for funding was based on the central hypothesis that brain-specific autoantibodies can be used to identify proteins that will serve as circulating biomarkers for the assessment of mild traumatic brain injury (TBI). The specific aims, which addressed the central hypothesis, covered both a laboratory rat (*rattus norvegicus*) animal model and a human tissue model. Within the context of this project, basic science research and discovery was carried out in the animal model and then translated to human TBI blood samples for validation.

GLOBAL AUTOIMMUNE PROFILING OF TBI-INDUCED AUTOANTIBODIES BY 1-DIMENSIONAL IMMUNOBLOTTING IN AN ANIMAL MODEL

Global autoimmune profiling was developed as an unbiased approach to investigate the expression of autoantibodies against each protein making up the brain proteome. We initially evaluated the effectiveness of this strategy using one-dimensional (1-D) immunoblotting. Specifically, rat brain proteome was fractionated on 1-D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to : polyvinylidene fluoride (PVDF) membrane and then probed with immunoglobulins present in serum of control or TBI rats. This investigation confirmed that TBI-induced autoantibodies could, in fact, be identified by 1-D immunoblotting. Moreover, the reactive protein features can be mapped to replicate protein gels and identified proteomically.

Figure 1 depicts immunoblots of rat brain proteome probed with control or TBI serum. Equal volumes and concentrations of membrane bound (lanes 1 and 2) and soluble proteomes (lanes 3 and 4) of a naïve rat brain were fractionated on 10% polyacrylamide gels and either

transferred to PVDF membranes or stained with Coomassie blue. Blots were probed with serum pooled from eight adult male naïve or eight adult male TBI rats (1:250 dilution). Reactive autoantibodies were visualized by horseradish peroxidase-labeled anti-rat IgG and enhanced chemiluminescence using a Fuji Image Reader LAS-3000 system and Multi Gauge V3.0 software. Immunoreactive features unique to the TBI blots were mapped to replicate Coomassie-stained protein gels and unique features were excised from the gel and identified by peptide mass finger printing. The results of this analysis included identification of alpha internexin, collapsin response mediator protein 2 (CRMP2) and the brain specific isoform of creatine kinase (CKBB) as candidate TBI biomarkers. Importantly, CKBB has been recognized as a potential TBI biomarker for more than two decades, indicating the validity of autoimmune profiling as a strategy for identifying TBI biomarkers that are novel to the field. In this regard, alpha internexin and CRMP2 are completely novel to the field of TBI biomarkers. Similar experiments investigating IgM autoantibodies yielded similar results (see Fig. 3).

Based on these promising results attention was placed on increasing the resolving power of our experimental approach. Due to the limited resolution of 1-D gel electrophoresis, complex samples often result in bands of interest containing more than one protein. A solution to this problem is the use of two-dimensional (2-D) fractionation on large-scale gels. This approach allows for the separation the brain proteome by both isoelectric point and molecular weight, thus effectively separating proteins with similar size or charge.

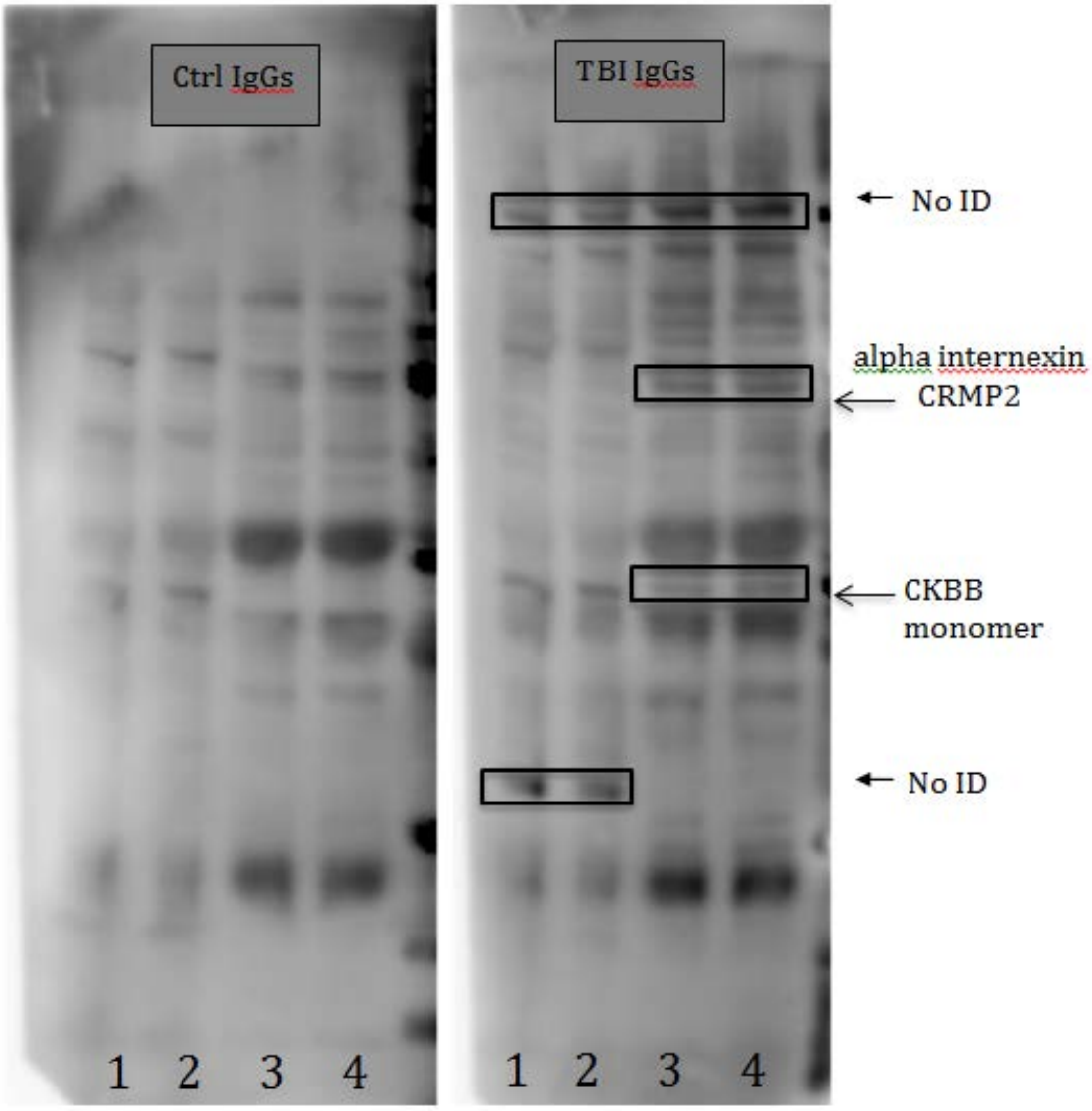

Figure 1. Autoimmune profiling of TBI-induced autoantibodies by 1-D gel electrophoresis and immunoblot analysis. The membrane bound (lanes 1 and 2) and soluble proteomes (lanes 3 and 4) of whole rat brain were fractionated on 10% polyacrylamide gels and transferred to PVDF. Blots were probed with serum pooled from eight naïve (left panel) or eight TBI (right panel) rats (1:250 dilution). Reactive autoantibodies were visualized by horseradish peroxidase-labeled anti-rat IgG or IgM and enhanced chemiluminescence. Reactive features that were unique to TBI (arrows) were mapped to replicate, Coomassie-stained protein gels and identified by peptide mass finger printing. No ID = no identification.

GLOBAL AUTOIMMUNE PROFILING OF TBI-INDUCED AUTOANTIBODIES BY 2-DIMENSIONAL IMMUNOBLOTTING IN AN ANIMAL MODEL

Based on the success of 1-D autoimmune profiling for the discovery of TBI-induced autoantibodies, a more intense search was carried out using 2-D gel electrophoresis and the largest gels commercially available. Soluble proteome of rat brain was first subjected to isoelectric focusing (1st dimension) and then molecular weight separation (2nd dimension). Next, the fractionated proteins were transferred to PVDF membranes and probed with serum pooled from eight naïve adult male Sprague Dawley rats or eight adult male TBI rats (CCI, 7 days, 1:250 dilution). Reactive autoantibodies were visualized by horseradish peroxidase labeled anti-rat IgG or IgM and enhanced chemiluminescence (Fig. 2). The control immunoblot (naïve) shows a characteristic pattern of IgG binding that reflects non-specific binding due to the relatively high concentration of serum (1:250 dilution) used to maximize the intensity of specific autoimmune signals. The primary focus of this investigation, however, was on immunologic signals that were newly revealed or greatly enhanced on TBI blots as compared to control blots. Boxes indicate areas on blots where differences between control and TBI were especially evident. Immunoreactive features unique to TBI were mapped to replicate silver stained protein gels. Unique protein features were excised from the gels and identified by peptide mass finger printing. In some cases, protein identifications were confirmed by tandem mass spectrometry performed at the W. M. Keck Biotechnology Resource Laboratory, Yale University.

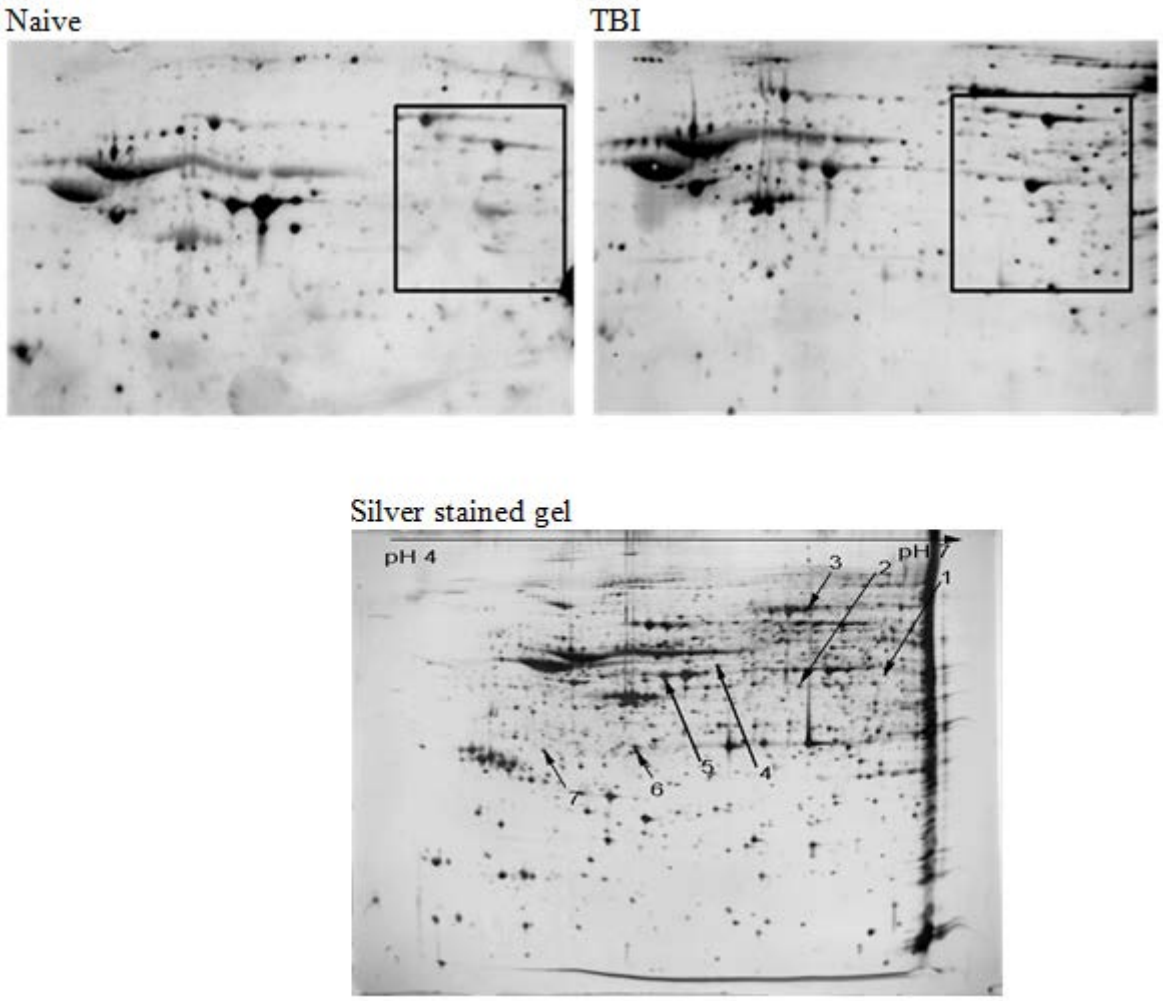

Figure 2. Autoimmune profiling of TBI-induced autoantibodies by 2-D gel electrophoresis and immunoblot analysis. The soluble proteome of whole rat brain (500 µg) was fractionated by isoelectric point (pH 4-7, 1st dimension) and molecular weight (2nd dimension), and then transferred to PVDF. The resulting immunoblots were probed with serum pooled from eight naïve (upper left panel) or eight TBI (upper right panel) rats (1:250 dilution). Reactive autoantibodies were visualized by horseradish-labeled anti-rat IgG or IgM and enhanced chemiluminescence. Boxes indicate areas on blots where differences between control and TBI were especially marked. Reactive features were mapped to replicate, silver stained protein gels (lower panel) and identified by peptide mass finger printing and/or tandem mass spectrometry. Protein identification were as follows: 1. TUC-4b; 2. dual specificity mitogen-activated protein kinase 1; 3. CRMP2; 4. neuronal pentraxin 1; 5. creatine kinase B-type; 6. mu-crystallin homolog; 7. annexin A5. The experiment presented here is representative of 14 separate runs analyzing different pools of control and TBI serum (autoantibodies) and involving 4 to 8 gels each.

CANDIDATE TBI BIOMARKERS IDENTIFIED BY GLOBAL AUTOIMMUNE PROFILING

A total of fourteen 2-D gel analyses were performed using pools of control and TBI serum prepared from different groups of rats. Each run consisted of 4-8 gels. After several iterations a number of candidate TBI biomarkers began to replicate, with both IgG and IgM screening. Proteins were selected as candidate TBI biomarkers based upon their repeated identification as targets for autoantibodies and by their reported localization and functions within the CNS. A composite Venn diagram of these identifications is presented below in Figure 3.

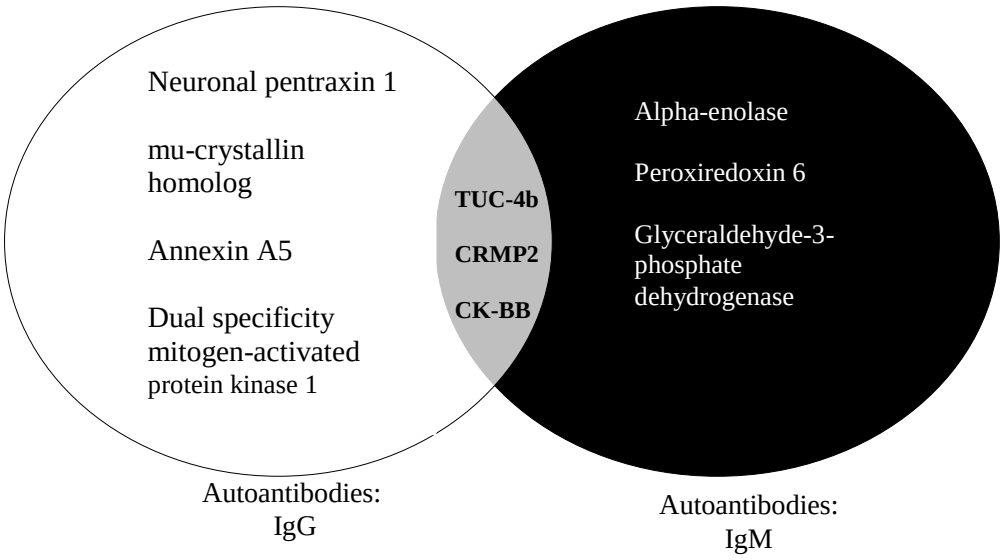

Figure 3. Venn diagram of candidate TBI biomarkers identified by global autoimmune profiling. Proteins identified by IgG autoantibodies are depicted on the left, proteins identified by IgM autoantibodies are depicted on the right and proteins identified by both IgG and IgM autoantibodies are depicted within the overlapping region in the center of the diagram.

A representative example of the TBI biomarker discovery process is presented in Figure 4 for the novel candidate, peroxiredoxin 6 (PRDX6). In the present project, the greatest success was achieved with the discovery and development of PRDX6. However, IEAs were similarly developed and used for the investigation of CKBB and CDK5 (CDK5 was discovered by protein microarray). Efforts to develop analytical tools for neuronal pentraxin 1, PCLO, CRMP2, and mu- crystallin homolog were met with limited success and did not yield IEAs suitable for the analyses needed here. This was due to either the absence of appropriate antibodies, standard proteins or a lack of assay sensitivity.

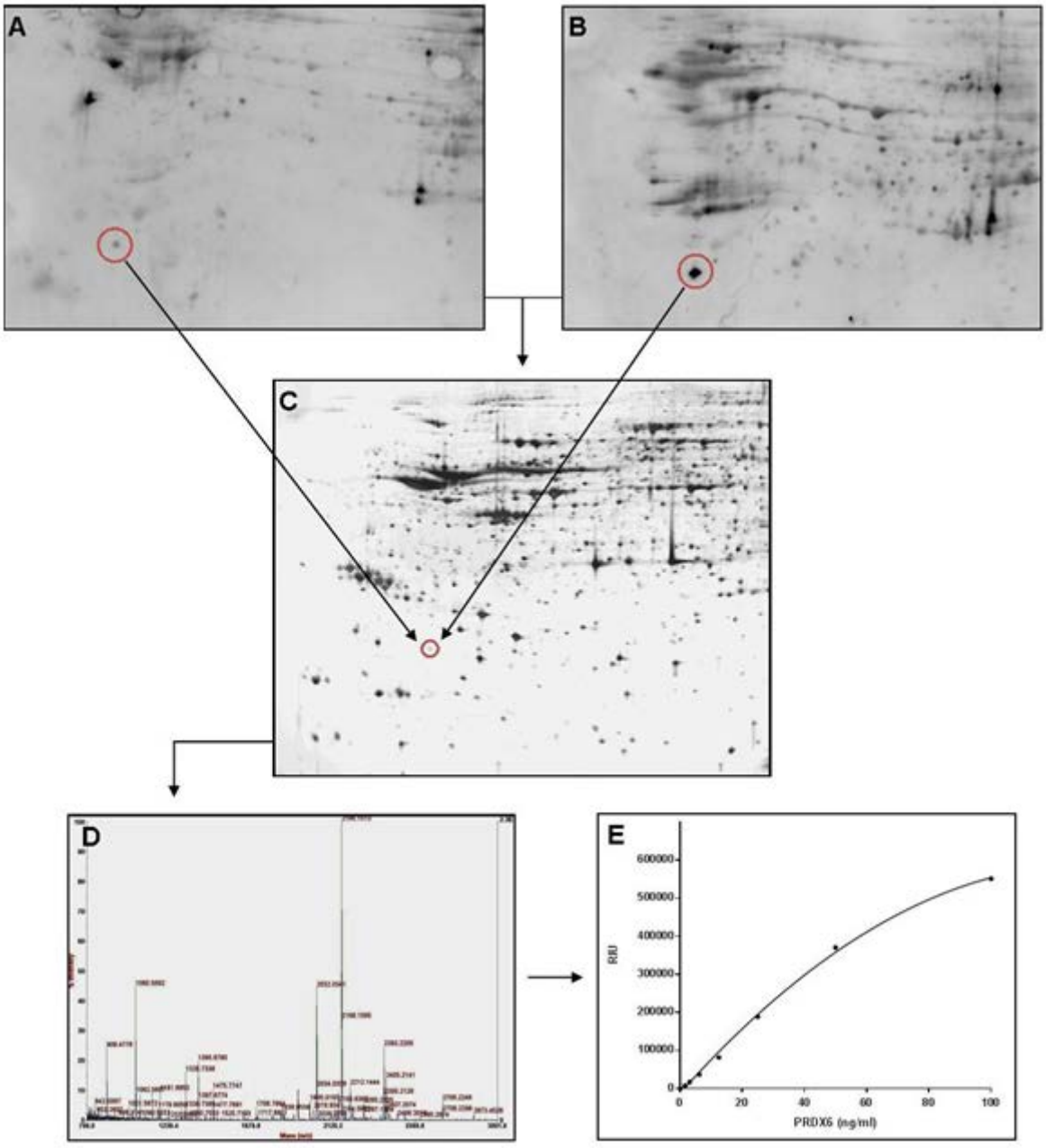

Figure 4. Discovery of PRDX6 as a candidate biomarker for brain injury. The rat brain proteome was fractionated by 2-D gel electrophoresis and transferred to PVDF. Blots were probed with serum from control and TBI rats (1:250), and visualized by enhanced chemiluminescence using pooled anti-rat IgG and IgM detection antibodies (panels A and B, respectively). A feature showing enhanced autoreactivity following TBI (circles) was mapped to a replicate protein gel (panel C) and identified by peptide mass finger printing as PRDX6 (panel D MALDI-TOF Spectra). Panel E presents a representative standard curve for the sandwich IEA developed to measure PRDX6 in human blood.

CHARACTERIZATION OF PRDX6 AS A CANDIDATE TBI BIOMARKER PROTEIN: IMMUNOREACTIVE PRDX6 IN MATCHED HUMAN SERUM AND PLASMA

The performance characteristics of the PRDX6 IEAs were evaluated using different types of biological samples including serum and plasma, as well as extracts of human brain and platelets. Similar tests performed with rat samples revealed that the PRDX6 IEA does not cross-react with this species.

Figure 6 presents the mean values for PRDX6 in human serum and plasma from normal male and female volunteers. The samples were prepared as matched sets from the same blood draws (n=10); NaEDTA was used as anticoagulant for the preparation of plasma. The data presented represent two separate experiments (panels A and B) carried out on two different sets of matched plasma and serum samples (N=40). Levels of PRDX6 were estimated using a standard curve that included an equivalent amount of either horse serum or chicken plasma (NaEDTA anticoagulant) to control for the non-specific effects of serum or plasma matrix, respectively. The presence of non-cross reactive (non-human) plasma or serum matrix increased nonspecific background signal by two- to three-fold over buffer alone, but did not interfere with the performance of recombinant PRDX6 protein standard in the IEA. Levels of PRDX6 in plasma were higher in females as compared to males ($p < 0.001$) in both experiments (168 ± 36 ng/mL vs. 65 ± 10 ng/mL and 178 ± 30 ng/mL vs. 43 ± 8 ng/mL, respectively). The process of coagulation dramatically reduced measured concentrations of PRDX6 in female blood samples ($p < 0.01$). A tendency for this phenomenon was also observed in male samples; however, the effect here was not significant due to the lower starting levels of PRDX6 in male plasma.

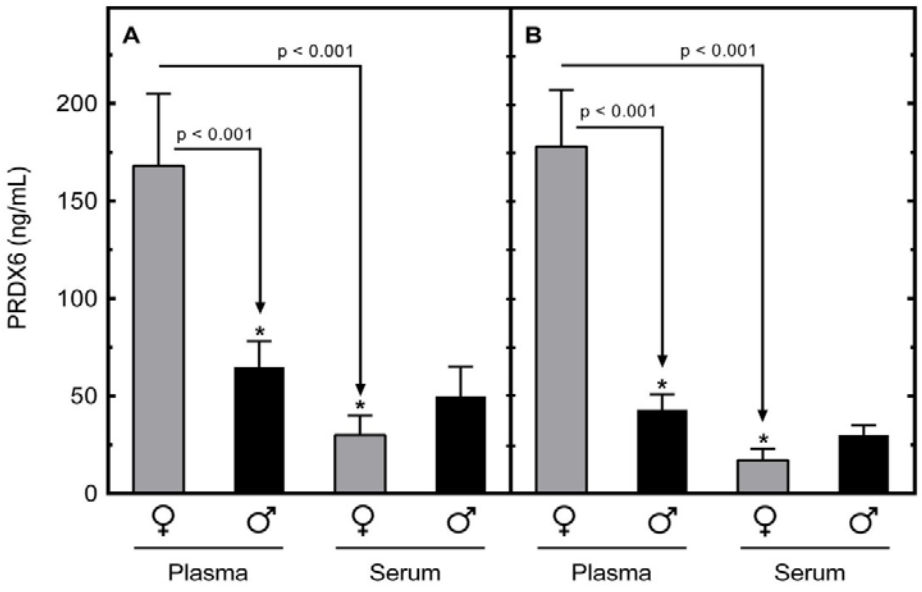

Figure 6. Comparison of levels of PRDX6 in human plasma and serum. Serum and plasma were prepared as matched sets from blood samples drawn from normal male and female volunteers (N=10 each). The experiment was replicated in a second independent cohort of the same size (panels A and B). Differences with statistical significance are shown by the arrows and corresponding p-values.

CHARACTERIZATION OF PRDX6 AS A CANDIDATE TBI BIOMARKER PROTEIN: IMMUNOHISTOCHEMISTRY

Immunohistochemistry on PRDX6 was performed to gain better insight into the neurobiology of this antioxidant enzyme. This preliminary study was performed in rat brain using an anti-PRDX6 antibody that recognizes rodent PRDX6 in fixed brain tissue. Brain expression of PRDX6 was examined in one naïve and one adult male CCI rat, 8 days post-injury. An analysis of cellular co-localization was carried out using anti-GFAP IHC to identify (Millipore), IBA-1 and ED-1 to identify quiescent and activated microglia respectively, and Neu N to identify neurons. In all cases, a donkey anti-rabbit green fluorescence antibody, Alexa Fluor 488 secondary antibody, was used to visualize PRDX6, whereas a donkey anti-mouse red

fluorescence antibody was used to visualize the cell-specific detection antibody. Secondary only controls carried out for each co-localization condition were all negative (not shown).

This study demonstrated that the expression of PRDX6 is predominantly in astrocytes, with little or no expression detected in microglia or neurons. A finding of particular note is the remarkable abundance of PRDX6 in astrocyte foot processes impinging on the walls of cerebral blood vessels (Fig. 7 and Fig. 8). Further, the expression of PRDX6 appeared to be up-regulated by TBI, creating a gradient of expression that was highest in the penumbra and lower in surrounding uninjured brain tissue (Fig. 9). Finally, the expression of PRDX6 was also detected in cell-like structures of uncharacteristic morphology located in the region of the penumbra. Based upon the IHC staining controls, these PRDX6-expressing structures appear to be necrotic astrocytes or possibly apoptotic remnants of astrocytes.

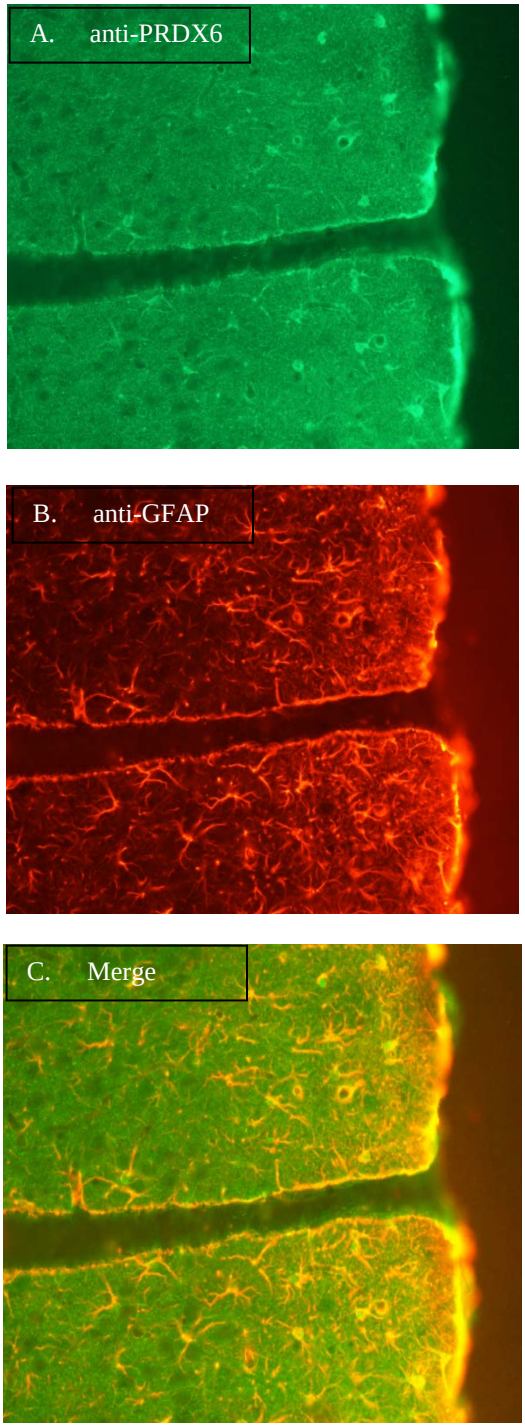

Figure 7. PRDX6 is highly expressed in astrocytes in rat cerebral cortex. The 40x section includes a blood vessel sectioned along its axis to reveal its wall and lumen. Panel A shows cells labeled by anti-PRDX6 antibodies. Panel B shows cells reactive with the astrocyte marker protein, GFAP. Panel C shows the merge of the green and red fluorescence to reveal the co-localization of PRDX6 and GFAP in astrocytes (yellow).

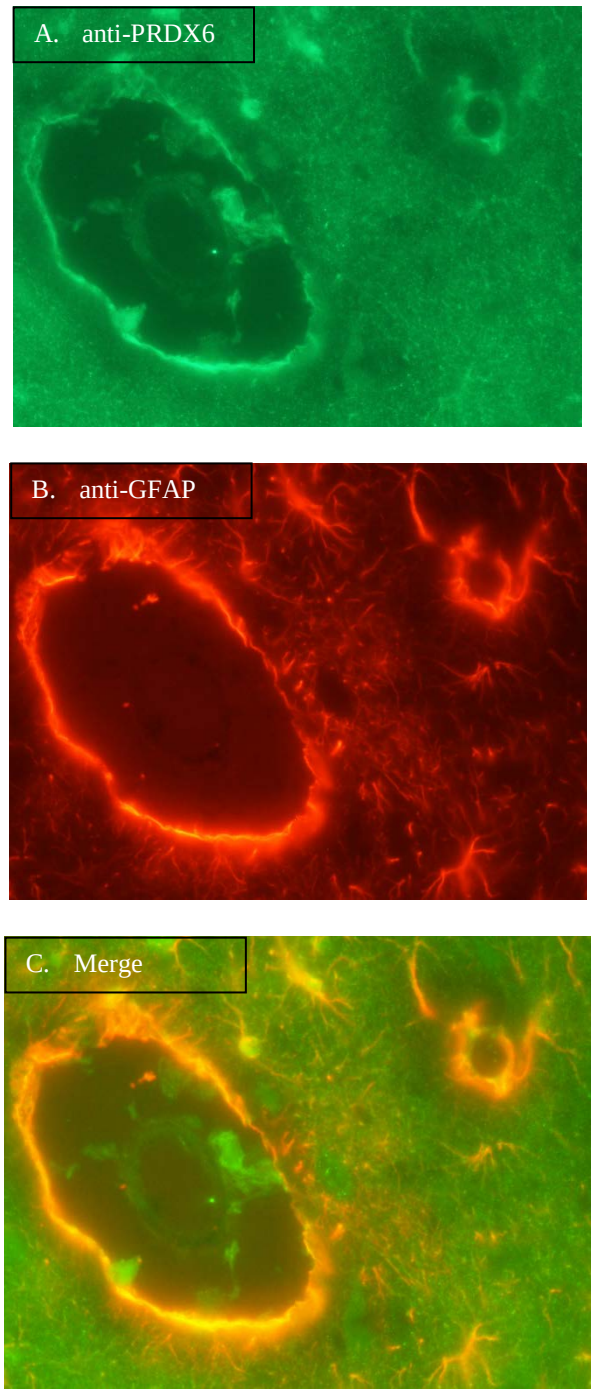

Figure 8. PRDX6 is highly expressed in astrocytes at the blood brain barrier in rat cerebral cortex. The 40x section includes two blood vessels cross-sectioned to reveal its wall and lumen, and in the larger vessel, a nucleated blood cell. Panel A shows cells labeled by anti-PRDX6 antibodies (green). Panel B shows cells reactive with the astrocyte marker protein GFAP (red). Panel C shows the merge of the green and red fluorescence to reveal the co-localization (yellow) of PRDX6 and GFAP in astrocytes with intense expression of PRDX6 at the brain-blood vessel interface.

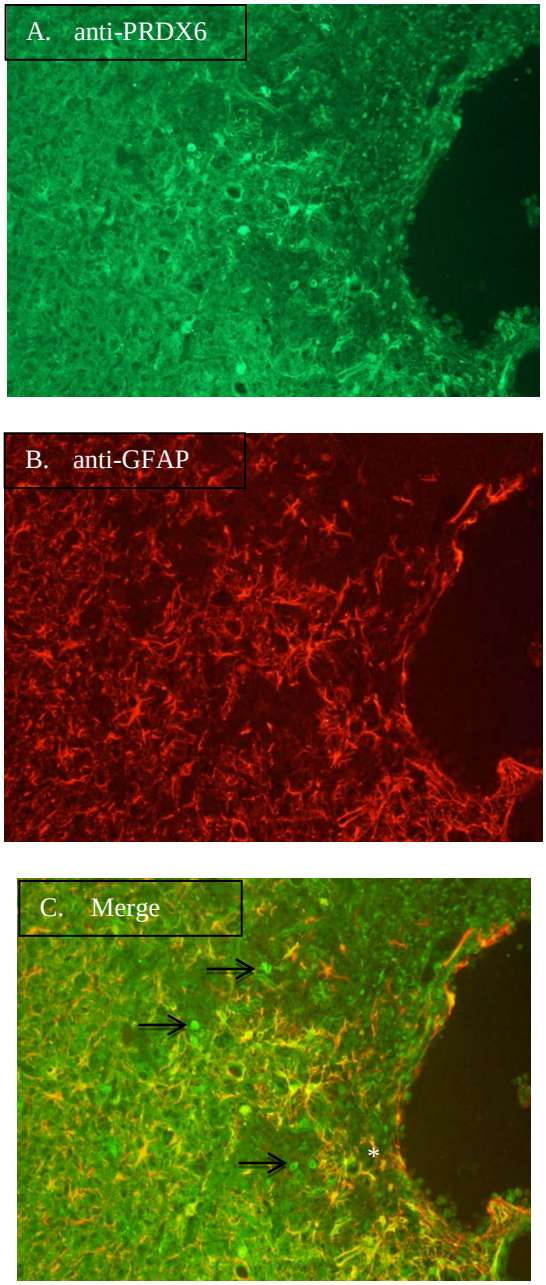

Figure 9. PRDX6 is highly expressed in astrocytes in rat cerebral cortex surrounding the penumbra. The sample was collected 8 days following TBI. The 10x section shows the lesion site on the right and includes a portion of the penumbra (*). Panel A shows cells labeled by anti-PRDX6 antibodies (green). Panel B shows cells reactive with the astrocyte marker protein, GFAP (red). Panel C shows the merge of the green and red fluorescence to reveal the co-localization (yellow) of PRDX6 and GFAP in astrocytes. There is an apparent gradient in PRDX6 expression from highest in the penumbra, as well as, an increased size of the PRDX6-expressing astrocytes in the penumbra. In addition, the presence of PRDX6 positive cells thought to be narcotic astrocytes are evident surrounding the lesion (arrows, panel C)

The remarkable abundance of PRDX6 in brain, its perivascular concentration and its apparent up-regulation in response to TBI all support the conclusion that PRDX6 will to be an informative biomarker for TBI.

CANDIDATE TBI BIOMARKERS IDENTIFIED BY AUTOIMMUNE PROFILING IN BOTH RODENTS AND HUMANS

Table 1 presents a list of the most promising candidate TBI biomarker proteins identified by global autoimmune profiling in rats or protein microarray profiling in humans. As noted previously, the proteins listed here were selected on the basis of their repeated identification as targets for TBI-induced autoantibodies and by their reported functions and specificity in brain. This list has been formally filed in an invention disclosure document.

Table 1. Candidate TBI Biomarker Proteins Identified by Autoimmune Profiling.

1	Alpha internexin – 66kDa A class IV neuronal intermediate filament protein involved in morphogenesis of neurons. Primarily expressed in adult CNS
2	Beta-soluble NSF attachment protein (betaSNAP) – 33kDa Mediates vesicular transport and release in neurons. Brain specific expression
3	Collapsin response mediator protein (CRMP2) – 62kDa Neuronal receptor for semaphorin, involved in guidance of growth cone. Neuron specific expression
4	Creatine kinase B (CK-BB) – 42kDa Cytosolic enzyme that catalyzes the formation of high energy phosphocreatine Predominately expressed in brain
5	CRMP4 (TUC-4b) – 62kDa Neuronal receptor for semaphorin, involved in guidance of growth cone. Neuron specific expression
6	Cyclin-dependent kinase 5 (CDK5)* – 33kDa Involved specifically in neuronal processes: migration, cortical layering, and synaptic plasticity. Dysregulation is linked to neuropathological alterations: CRMP2 and tau hyperphosphorylation, neuronal and synaptic loss Highly expressed in mature neurons

7	Mu-crystallin homolog – 33kDa Binds thyroid hormone and catalyzes reduction of imine bonds in proteins Expressed in brain and lens of the eye
8	Neuronal pentraxin-1 – 47kDa Involved in synaptic remodeling. Known as the C-reactive protein of brain. Brain specific expression
9	Peroxiredoxin 6 (PRDX6)– 25kDa Antioxidant enzyme that catalyzes the hydrolysis of H_2O_2 Highly expressed in brain, also expressed in lung and kidney
10	Presynaptic cytomatrix protein (piccolo, PCLO)* - 552kDa Presynaptic cytoskeletal matrix protein that mediates the release of synaptic vesicles Neuron specific expression
11	RalA-binding protein 1 (RALBP1)* - 76kDa Interacts with GTP binding proteins to regulate receptor mediated endocytosis Widely expressed in many cell types
12	Regulator of G-protein signaling 8 (RGS8)* - 21kDa Inhibits GTPase signaling transduction Brain specific expression
	*Identified by protein microarray in humans, all others by immunoprofiling in rat

DEVELOPMENT OF IMMUNOSORBENT ELECTROCHEMILUMINESCENCE ASSAYS (IEA) FOR CREATINE KINASE BB AND CYCLIN-DEPENDENT KINASE 5

IEAs were successfully developed for creatine kinase BB and cyclin-dependent kinase 5 following the approaches detailed for PRDX6. Both assays were used for the analysis of human (Table 5 and Figure 14 – CDK5 and CKBB not shown) and rodent samples (not shown). As noted above, dedicated efforts to develop assays for neuronal pentraxin 1, CRMP2, mu crystallin homolog and presynaptic cytomatrix protein were not successful due to the lack of suitable antibodies available commercially. We did not attempt to develop assays for the other candidates listed in Table 11 due to the limitations of time and/or reagents.

APPROACHES FOR THE ANALYSIS OF CANDIDATE BIOMARKERS IN HUMAN PATIENT SAMPLES

The analysis of human samples was designed to identify a biomarker signature consisting of a profile of proteins that would effectively differentiate the spectrum of TBI severity spanning from no injury, to mild/moderate injury, to severe injury. These analyses involved the use of a multiplex platform (for measuring 6 candidates simultaneously) and singleplex analyses. The analytes measured by the multiplex platform were: S100b, BDNF, NSE, MCP1/CCL2, ICAM-5 and GFAP. Singleplex assays were used for the measurement of PRDX6, CDK5.

ANALYSIS OF CANDIDATE BIOMARKERS IN HUMAN PATIENTS EXPERIENCING MILD TO MODERATE TBI

This study determined the response pattern of a panel of candidate biomarkers in 154 TBI adult patients admitted into local emergency departments with apparent brain injury. The majority of the patients in this study were admitted into Suburban Hospital, Bethesda, MD. The mechanisms of injury were mainly divided among injuries due to falls (54%), direct head impact (27%) and acceleration/deceleration injuries (mostly automobile accidents, 12%). The average Glasgow Coma Scale score at the time of admission was above 14.5, consistent with mild TBI. Clinical data also included information concerning loss of consciousness, post-traumatic amnesia, computed axial tomography (CAT or CT) scan and magnetic resonance imaging (MRI). An analysis of the CT and MRI data revealed that 30%, 39%, 24% of the TBI patients presented with CT, MRI or CT and MRI imaging abnormalities, a feature consistent with the diagnosis of moderate TBI. Nevertheless, these same imaging positive patients had normal or near-normal Glasgow Coma Scale scores, and loss of consciousness and post-traumatic amnesia intervals consistent with mild TBI. Together, these observations illuminate the difficulty that exists in making a definitive diagnosis of mild TBI based upon conventional modalities for assessment.

The clinical component of this study was designed and directed by Dr. Lawrence Latour with the primary objective of evaluating the limits of current imaging technology in diagnosing mild TBI. Accordingly, blood samples were collected at the time of admission into an emergency department and then again at a time ranging between two and seven days after the first sample collection. The variation in the timing for the second blood sample reflects the clinical schedule for neuroimaging. Commercially purchased control samples were collected from normal volunteers (76% Caucasian) ranging in age from 19-50, and with a mean and median age of 25 and 24 respectively. These samples were considered most relevant to our research design compared samples from other emergency room patients admitted with non-TBI injuries. It would be difficult to establish baseline values for our candidate biomarkers in non-TBI injured patients, considering that an emergency room admission by its very nature disqualifies those patients as healthy controls.

A listing of the available patient demographics and clinical data is presented below in Table 2. A complete listing of all clinical variables collected is available. Controls listed in Table 2 are a representative subset and those used in the data analysis model depicted in Figure 12.

Table 2. Demographic characteristics and clinical variables for patients with mild TBI

		TBI N = 154	**Controls N = 30**
Age			
	Mean, y (SD)	47 (19)	25 (5)
	Median	45.8	24
	Range	19-91	19-50
Gender (%)			
	Male	103 (67)	15 (50)
	Female	43 (28)	15 (50)
	Unknown	8 (5)	0 (0)

Race (%)			
	Caucasian	103 (67)	23 (76)
	Non-Caucasian	30 (19)	7 (24)
	Unknown	21 (14)	0 (0)
Education (%)			
	< Grade 12	4 (3)	
	High School / equivalent /Associates	70 (45)	
	Bachelor's degree	27 (17)	
	PhD/Professional	30 (19)	
	Unknown	23 (15)	30 (100)
Mechanism of Injury (%)			
	Acceleration/deceleration	18 (12)	0
	Fall	83 (54)	0
	Direct impact	43 (27)	0
	Unknown	10 (6)	0
Glasgow Coma Scale score in ED (%)			
	< 9	3 (2)	0
	9 - 12	3 (2)	0
	≥ 13	132 (86)	30 (100)
	Unknown	16 (10)	0
Loss of Consciousness (%)			
	Yes	69 (45)	0
	No	58 (37)	30 (100)
	Unknown	27 (18)	0
Post-traumatic amnesia (%)			
	Yes	83 (54)	0
	No	67 (43)	30 (100)
	Unknown	4 (3)	0
Imaging - CT (%)			
	CT-Scalp_Hematoma	34 (22)	0
	CT-Skull_Fracture	1 (0.6)	0
	CT-Subdural_Hematoma_Acute	16 (10)	0
	CT-Subarachnoid_Hemorrhage	24 (16)	0
	CT-Contusion	11 (7)	0
	CT-Intracerebral_Hemorrhage	10 (6)	0
	CT-Diffuse Axonal Injury	0 (0)	0
	CT-Intraventricular_Hemorrhage	4 (3)	0

Imaging - MRI (%)			
	MRI-Scalp_Hematoma	24 (16)	0
	MRI-Epidural_Hematoma	0 (0)	0
	MRI-Subdural_Hematoma_Acute	21 (14)	0
	MRI-Subarachnoid_Hemorrhage	23 (15)	0
	MRI-Contusion	19 (12)	0
	MRI-Intracerebral_Hemorrhage	20 (13)	0
	MRI- Diffuse Axonal Injury	10 (6)	0
	MRI-Intraventricular_Hemorrhage	9 (6)	0
Admitted to Hospital (%)		100 (65)	0
	Unknown	32 (20)	
Neurobehavorial symptom inventory score			N/A
22 symptoms scoring 0-4	Mean	0.75	
Extended Outcome 30-day Post-injury			N/A
Satisfaction with life scale (1-35) (n=26)	Mean	23	
Glasgow outcome scale extended (1-8) (n=59)	Mean	6.5	
Extended Outcome 90-day Post-injury			N/A
Satisfaction with life scale (1-35) (n=26)	Mean	24	
Glasgow outcome scale extended (1-8) (n=61)	Mean	7	

EFFECTS OF MILD TO MODERATE TBI ON PLASMA LEVELS OF CANDIDATE BIOMARKERS PROTEINS

The patient samples were analyzed as two cohorts of approximately equal size. Table 3 presents the mean plasma levels for all biomarkers at all-time points for the 154 TBI patients and 30 commercially purchased controls making up this study. All controls were assayed with each TBI cohort with equivalent results. Values that are statistically different from control values are indicated by specific p-values.

Table 3. Mean plasma values of candidate TBI biomarker proteins in patients with mild to moderate TBI and controls.

Condition	ng/ml	BDNF	GFAP	ICAM -5	MCP1/ CCL2	NSE	S100b	PRDX6
Control	Mean	2.78	<0.30	0.90	0.11	29.95	0.15	78.20
	SEM	0.56	-	0.06	0.01	6.22	0.03	17.51
	Mean	9.08	<0.30	0.92	0.17	55.50	0.72	387.9
Baseline	SEM	0.58	-	0.02	0.01	2.83	0.13	14.95
	p =	<0.0001	-	NS	<0.001	<0.0001	<0.03	<0.0001
	Mean	9.41	<0.30	0.90	0.17	67.34	0.62	430.8
2-7 Days	SEM	0.64	-	0.02	0.01	3.32	0.12	15.81
	p =	<0.0001	-	NS	<0.001	<0.0001	<0.03	<0.0001

Figure 10 presents additional information on the effects of mild to moderate TBI on plasma levels of PRDX6. Data shown are for male and female patients at the time of admission to an emergency department and at a second time ranging from 2 to 7 days post-injury. As depicted in the controls, plasma levels of PRX6 tended to be higher in females, as compared to males and levels in both males and females at admission were approximately 3-fold higher than control values. Accordingly, PRDX6 appears to be a sensitive indicator of mild to moderate TBI in both genders.

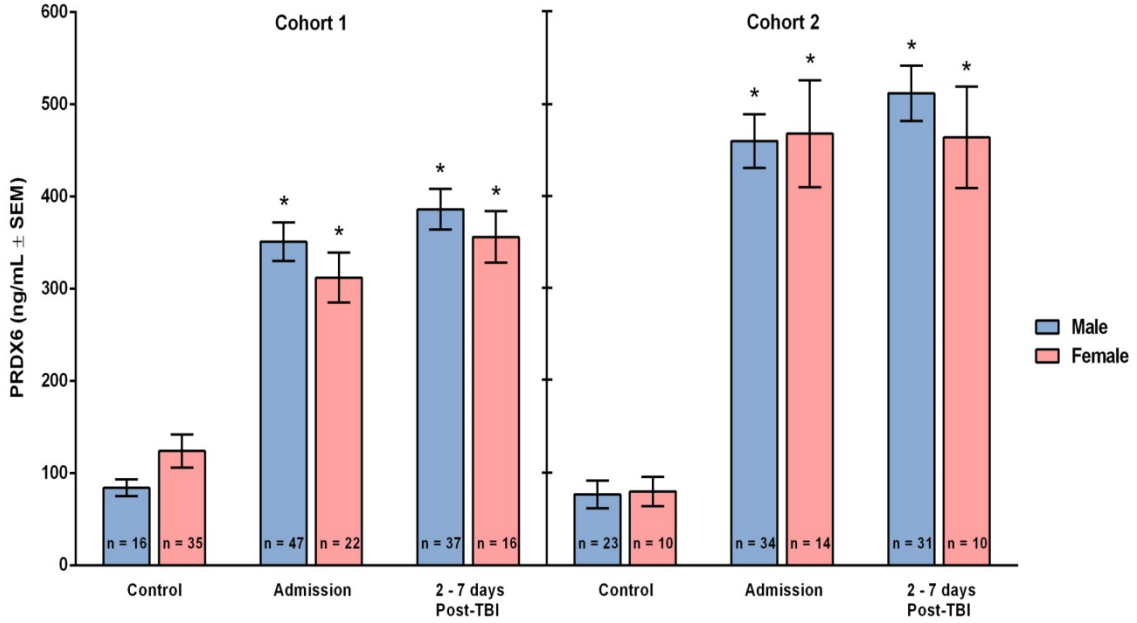

Figure 10. Comparison of levels of PRDX6 in human plasma from control and TBI patients at the time of admission and at 2-7 days post-injury. The experiment was replicated in a second cohort of similar size (panels Cohort 1 and 2). Bars reflect mean ± standard error. Sample sizes are listed within bar. * $p < 0.0001$ as compared to control values.

Figure 11 shows the time course for the fold-changes in plasma levels of seven candidate TBI biomarker proteins. Plotting data as a fold-change standardizes for the wide differences that exist in the absolute concentrations of the biomarkers in plasma. While the duration between the first and second samples was variable due to the study's design (see above), time-related increases in plasma levels were observed for five of the seven candidate biomarker proteins: BDNF, MCP1/CCL2, NSE, S100b, and PRDX6. Levels of GFAP were below the level of quantification for all time points and thus, no changes were detected under the conditions of this study.

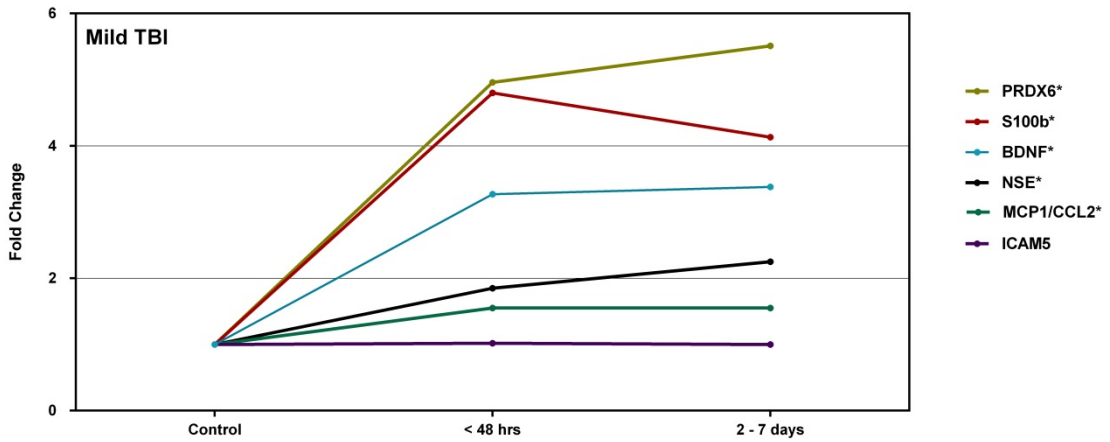

Figure 11. Effects of mild to moderate TBI on plasma levels of candidate biomarker protein expressed as fold-changes from controls values. Plotted are the values obtained within 48 hours of injury and again at 2 to 7 days post mild TBI. Graph lines for ICAM-5 and GFAP closely overlap. *Changes significantly different from control (see Table 2 for p-values)

FURTHER ANALYSIS OF PLASMA LEVELS OF CANDIDATE TBI BIOMARKERS IN PATIENTS WITH MILD TO MODERATE TBI

Data from the present study on mild to moderate TBI were further analyzed as depicted by the data analysis model presented in Figure 12. The examination was undertaken utilizing 7 dependent variables against 1, two-level (control:injured) independent variable. Multivariate analysis of variance (MANOVA) was used to minimize type I errors. It was recognized that the sample sizes were unequal, contained few outliers and displayed a skewed data distribution. The Box text and Levene's test which measure quality of covariance and variance respectively were both violated. After taking all of these conditions into account and considering the large sample size, a method I, type 3 sum of squares model design was used to keep all cells equally important. To further address the violation of statistical assumptions, data was analyzed by the following alternative means: analysis of variance, analysis of covariance, type II sum of squares, and transformation of data (log). A report on the Pillai's trace statistics for all calculations

undertaken in the research is available. Despite the uneven sample sizes, the "n" was sufficiently large in both studies that the results were still robust to violations of the statistical assumptions [1]. Therefore, a MANOVA analysis was utilized to determine how the demographic variables of age, race, gender and level of education might be related to biomarker responses. A similar analysis was performed to examine how the presence of abnormal medical imaging or Glasgow Coma Scale score might relate to biomarker responses.

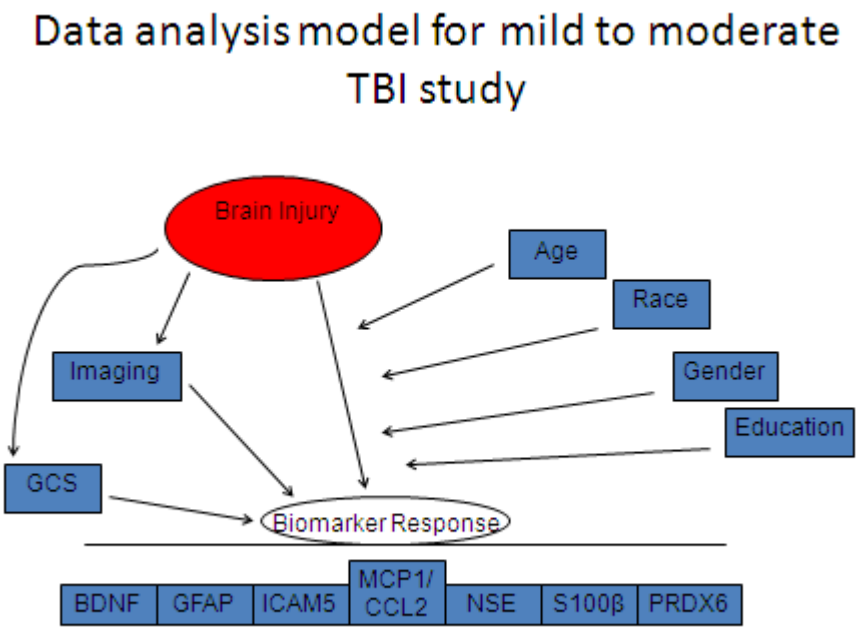

Figure 12. Data analysis model for the clinical and demographic data of the mild to moderate TBI study.

When co-varying for gender, race, age, and education, there was no overall difference between males and females and no differences with regard to race and education. With regards to age, younger patients (< 45 yrs) had modestly lower levels of MCP1/CCL2 at admission as compared to levels measured in older (> 45 yrs) individuals ($p > 0.05$). None of the other biomarkers showed this pattern. Regarding the two clinical variables examined, we could not

differentiate between levels of admission Glasgow Coma Scale or CT/MRI imaging positive results and changes in the levels of plasma biomarkers. Because the clinical variables were analyzed as nominal/dichotomous variables, and multiple dependent variables were considered a correlation analysis was not appropriate. In regards to the mild to moderate brain injury and biomarker response it was found that in every case the overall multivariate model was significant for each time point with an effect size greater than 0.14 and a power of 1.0.

ANALYSIS OF CANDIDATE BIOMARKERS IN HUMAN PATIENTS EXPERIENCING MODERATE TO SEVERE TBI

A study of moderate to severe TBI was conducted in collaboration with Dr. Shawn Rhind and his colleagues in the Defence Research and Development Canada, the Canadian counterpart to the Department of Defense. This study involved a total of 106 patients who were admitted with isolated head injuries and diagnosed with moderate to severe TBI. Plasma samples were obtained at admission and 6, 12, 24 hours post-injury. Glasgow Coma Scale scores on admission were all below 12 (range = 3-12; mean = 6; median = 6) and 26 of 106 patients died within the 24 hours following admission.

DEMOGRAPHIC DATA OF THE MODERATE TO SEVERE TBI PATIENTS

Table 4 lists the demographic and clinical data available for the subjects involved in this ongoing study. The 44 control samples were provided by our collaborator. They were described as non-trauma, non-post-traumatic stress disorder military personnel. The demographic and clinical data available from this study are currently limited as compared to the data recorded in the mild to moderate TBI study (discussed above). Accordingly, an in-depth analysis of these variables could not be undertaken here.

Table 4. Demographic characteristics and clinical variables for patients with moderate to severe TBI study.

		TBI	Controls
		Total, N = 106	Total, N = 44
Age			
	Mean, y (SD)	47 (21)	
	Median	45.5	
	Range	16-96	
	Unknown	0	44 (100)
Gender (%)			
	Male	85 (80)	
	Female	21 (20)	
	Unknown	0	44 (100)
Glasgow Coma Scale score in Emergency Department (%)			
	< 9	75 (71)	0
	9 - 12	28 (26)	0
	≥ 13	0 (0)	44 (100)
	Unknown	3 (3)	0
Marshall Score (%)*			
	I	15 (14)	0
	II	51 (48)	0
	III	9 (8)	0
	IV	18 (17)	0
	V	9 (8)	0
	VI	0	0
	Unknown	4 (4)	0
Surgery in first 24 hours (%)**			
	Yes	28 (26)	0
	No	78 (74)	0
	Unknown	0	0
Dead (%)		26 (24)	0
Glasgow outcome scale extended at Hospital Discharge(1-8) (n=27)***	Mean	3.2	0

*A rating scale with 6 categories, used to predict both the risk of increased intra-cranial pressure and outcome in adults (Category I = diffuse injury, no visible pathology-Category 6 = major CT abnormality [2].

** A description of the surgeries performed is available

*** A rating scale with 8 categories used to measure outcome and clinical status 6 months after injury. (1 = severe disability and poor outcome - 8 = highly functional and good outcome [3].

EFFECTS OF MODERATE TO SEVERE TBI ON PLASMA LEVELS OF CANDIDATE BIOMARKER PROTEINS

The patient samples were obtained as two cohorts of approximately equal size. Data from all the subjects at the individual time points was averaged prior to analysis. Statistical analysis (MANOVA) was similar to that utilized in the mild to moderate study. A report on the Pillai's trace statistics for all calculations undertaken in the research is available. Table 5 presents the mean plasma levels for all biomarkers at all-time points for TBI patients and controls making up this study. Values that are statistically different from control values are indicated by specific p-values.

Table 5. Plasma levels of candidate TBI biomarker proteins in patients with severe TBI.

Condition	ng/mL	BDNF	GFAP	ICAM-5	MCP1/ CCL2	NSE	S100b	PRDX6 Cohort 1 only	CDK5 Cohort 1 only
Control	Mean	1.18	<0.30	1.37	0.13	10.53	0.29	144.54	24.79
	SEM	0.22	-	0.08	0.01	0.89	0.03	24.20	15.04
	Mean	4.53	3.84	1.10	0.39	119.8	2.46	762.87	<6.80
Adm	SEM	0.54	1.49	0.14	0.05	9.95	0.58	85.45	-
	p =	<0.001	-	<0.08	<0.001	<0.001	<0.001	<0.001	-
	Mean	3.98	0.91	1.02	0.29	110.8	0.57	510.52	<6.80
6 hrs	SEM	0.41	0.15	0.08	0.05	9.28	0.09	53.15	-
	p =	<0.001	-	<0.001	<0.001	<0.001	<0.002	<0.001	-
	Mean	3.26	0.86	0.96	0.24	100.4	0.59	413.18	<6.80
12 hrs	SEM	0.43	0.14	0.07	0.03	9.26	0.12	43.53	-
	p =	<0.001	-	<0.001	<0.001	<0.001	<0.01	<0.001	-
	Mean	2.30	0.69	0.99	0.25	69.22	0.58	280.01	<6.80
24 hrs	SEM	0.25	0.06	0.08	0.03	7.35	0.17	44.44	-
	p =	<0.001	-	<0.001	<0.005	<0.001	<0.07	<0.006	-

Figure 13 presents the effects of moderate to severe TBI on plasma levels in cohort 1 of PRDX6. As shown here, at the time of admission circulating levels of PRDX6 were greater than 5-fold of control values. This increase in plasma PRDX6 exceeded the 4.9 fold increase observed

in the response to mild to moderate TBI (Fig. 11 and Table 3). Plasma levels of PRDX6 progressively declined over the next 6, 12, and 24 hours, but were still significantly elevated over controls at the 24-hour time point.

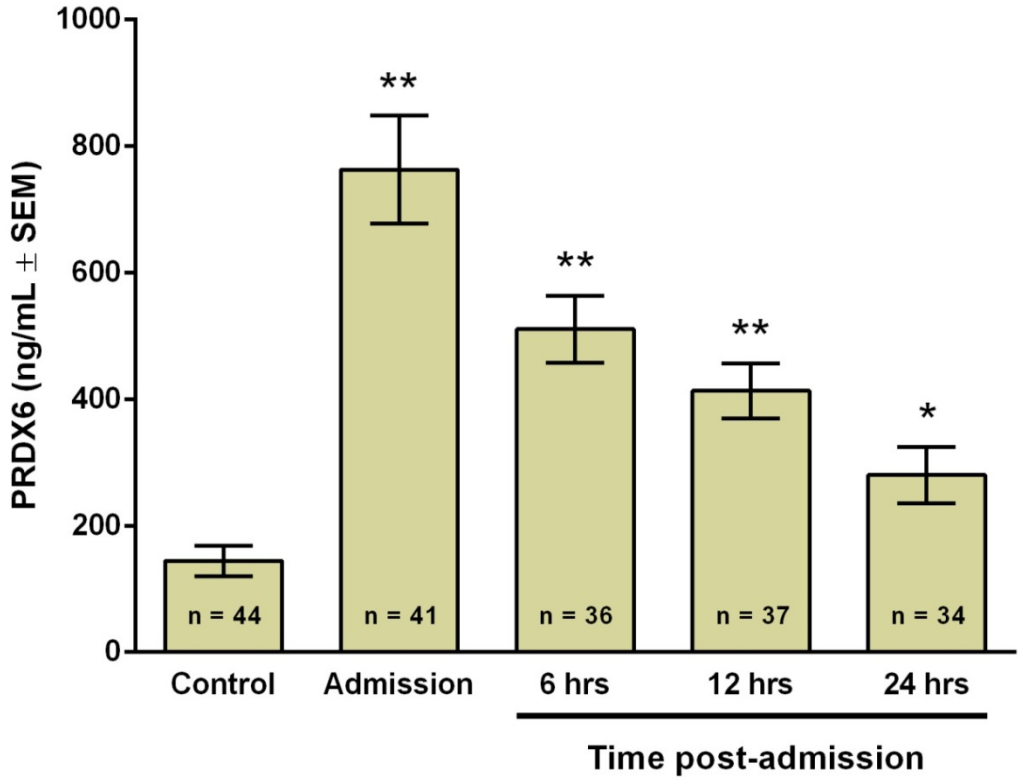

Figure 13. Time course for changes in plasma levels of PRDX6 in cohort 1 following moderate to severe TBI. Bars reflect mean ± standard error. Sample size listed within bar. ** p <0.0001, * p < 0.001.

Figure 14 shows the time course for the fold-changes in plasma levels of eight candidate TBI biomarker proteins. Time-related increases in plasma levels were observed for five of the eight candidate biomarker proteins: PRDX6, S100b, BDNF, NSE, and MCP1/CCL2. Levels of ICAM-5 were not appreciably altered under the conditions of this study. While the mean levels of GFAP in the TBI group rose into the detectable range following moderate to severe TBI, a fold change could not be calculated because control values were below the limit of detection.

Intriguingly, plasma levels of CDK5 were reduced in patients with moderate to severe TBI; however, this response could not be quantitated due to the low basal levels of plasma CDK5 in controls and the reduction in these low levels to values below the limit of assay quantification following TBI.

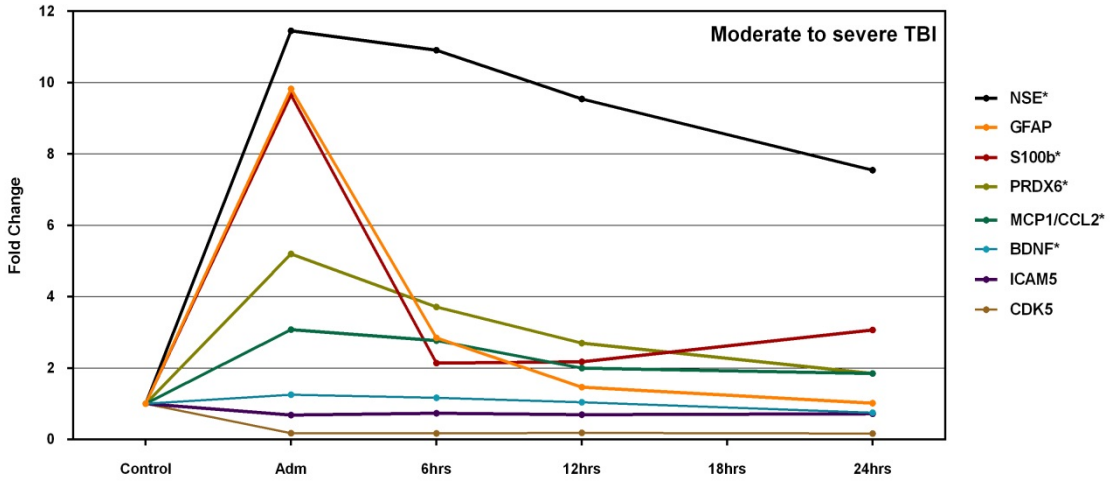

Figure 14. Effects of moderate to severe TBI on plasma levels of candidate biomarker proteins expressed as fold-changes from control values.

FORMULATION OF A TBI ASSESSMENT SCORE BASED UPON THE PROFILE OF BIOMARKER RESPONSES TO TBI

The distinctive profiles observed in plasma levels of biomarker proteins following TBI suggested that these patterns might contribute to formulation of a biomarker signature for the diagnosis of TBI. To explore this possibility, fold changes in the levels of each biomarker were calculated for samples collected at the time of hospital admission, as compared to control values. Table 8 presents the data from Table 5 for the study focusing on patients with mild to moderate TBI at the time of admission.

Table 6. Plasma values and fold changes of candidate TBI biomarker proteins in patients with mild to moderate TBI.

	BDNF	GFAP*	ICAM-5	MCP1/ CCL2	NSE	S100b	PRDX6
Control	2.78 ± 0.56	<0.30	0.90 ± 0.06	0.11 ± 0.01	29.95 ± 6.22	0.15 ± 0.03	78 ± 17.51
TBI Admission	9.08 ± 0.58	<0.30	0.92 ± 0.02	0.17 ± 0.01	55.50 ± 2.83	0.72 ± 0.13	388 ± 14.95
Fold Change	3	?	No Δ	2	2	5	5

*GFAP levels were beneath the level of detection in both control and TBI group.

A similar analysis was carried out for the data from patients diagnosed with moderate to severe TBI due to isolated head injury. Table 7 presents mean plasma levels and fold-changes for plasma levels of biomarker proteins in patients with moderate to severe TBI. In cases where changes were observed, these were all greater than those observed in response to mild TBI. While the mean levels of GFAP in the moderate to severe TBI group rose into the detectable range following injury, a fold-change could not be calculated because most of the control values (75%) were below the limit of quantification (0.3 ng/mL). Moreover, the increase in plasma concentrations of GFAP to a mean of 3.6 ± 1.1 ng/mL (median 0.8 ng/mL) measured in the injured patients reflects very high values in only 6 of 91 individuals who were disproportionally responsive as compared to the other 85 individuals in the group. In these highly responsive individuals, plasma values of GFAP ranged from 24 to 55 ng/mL (mean ± SEM: 40 ± 5 ng/mL). Accordingly, it is not possible to calculate a fold-change value for GFAP in response to TBI. However, it should be noted in that within individual patients a dramatic increase in plasma GFAP following TBI may be relevant to the diagnosis and assessment of their particular brain injury.

Table 7. Mean plasma values of candidate TBI biomarker proteins in moderate to severe TBI patients and their respective fold changes compared to controls.

	BDNF	GFAP**	ICAM-5	MCP1/CCL2	NSE	S100b	PRDX6*
Control	1.18 ± 0.22	< 0.30 -	1.37 ± 0.08	0.13 ± 0.01	10 ± 0.89	0.29 ± 0.03	145 ± 24
TBI Admission	4.53 ± 0.54	3.84 ± 1.49	1.10 ± 0.14	0.39 ± 0.05	120 ± 10	2.46 ± 0.58	763 ± 85
Fold Change	4	?	?	3	11	9	5

***** PRDX6 data represents patients from Cohort 1 only. ** No fold change could be calculated for GFAP because approximately 75% of the data for control values were beneath the level of quantification. This is in contrast to the TBI patients where, at admission, plasma GFAP values were all greater than the assay limit of quantification (0.3 ng/mL).

TBI ASSESSMENT SCORE

On the basis of these findings, we propose that a clinically relevant TBI Assessment Score can be based upon the fold changes observed in 5 of the biomarker proteins studied here. Table 8 shows the formulation of this score, which is simply the summation of the fold-changes observed in plasma levels of PRDX6, NSE, S100b, BDNF, and MCP1/CCL2. Because control levels are unchanged for these biomarkers, they each have been assigned a value of one, for a summation score of five. In the case of mild TBI, we calculated a summation score of 17 for the candidate biomarkers listed in Table 8. In the case of moderate to severe TBI the summation score was 32. Calculated in this fashion, the TBI Assessment Score clearly distinguishes mild TBI from controls and from moderate to severe TBI.

Table 8. Formulation of a TBI Assessment Score.

Protein	Control	Mild to Moderate	Moderate to Severe
BDNF	1	3	4
MCP1/CCL2	1	2	3
NSE	1	2	11
S100b	1	5	9
PRDX6	1	5	5
TBI Score	5	17	32

It is proposed that this strategy of multivariate analysis can be further developed for the improved diagnosis of mild TBI. The present data demonstrate the five biomarkers studied here can be used to establish a meaningful signature that readily identifies mild TBI with an objective and quantifiable assessment score. As depicted in Figure 15, this TBI Assessment Score complements and extends that of the Glasgow Coma Scale, which was originally designed for documenting states of consciousness, and therefore pertains best to conditions of moderate and severe TBI [4]. The benefit of the TBI assessment score proposed here is its ability to aid in the diagnosis of mild TBI. In addition to the increased diagnostic sensitivity, the TBI Assessment Score has a wider dynamic range than the Glasgow Coma Scale. Because the TBI Assessment Score is based upon definitive measures of circulating biomarkers, it is an objective assessment that is easily standardized across clinical settings.

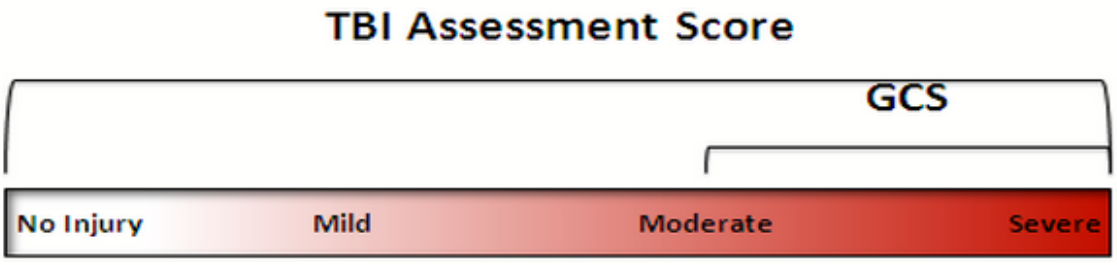

Figure 15. The Glasgow Coma Scale was designed to assess levels of consciousness. Accordingly, its clinical use focuses on the more severe forms of TBI. The TBI Assessment Score (TBI Score) proposed here has a wide dynamic range of sensitivity and can be used to assess the entire spectrum of TBI.

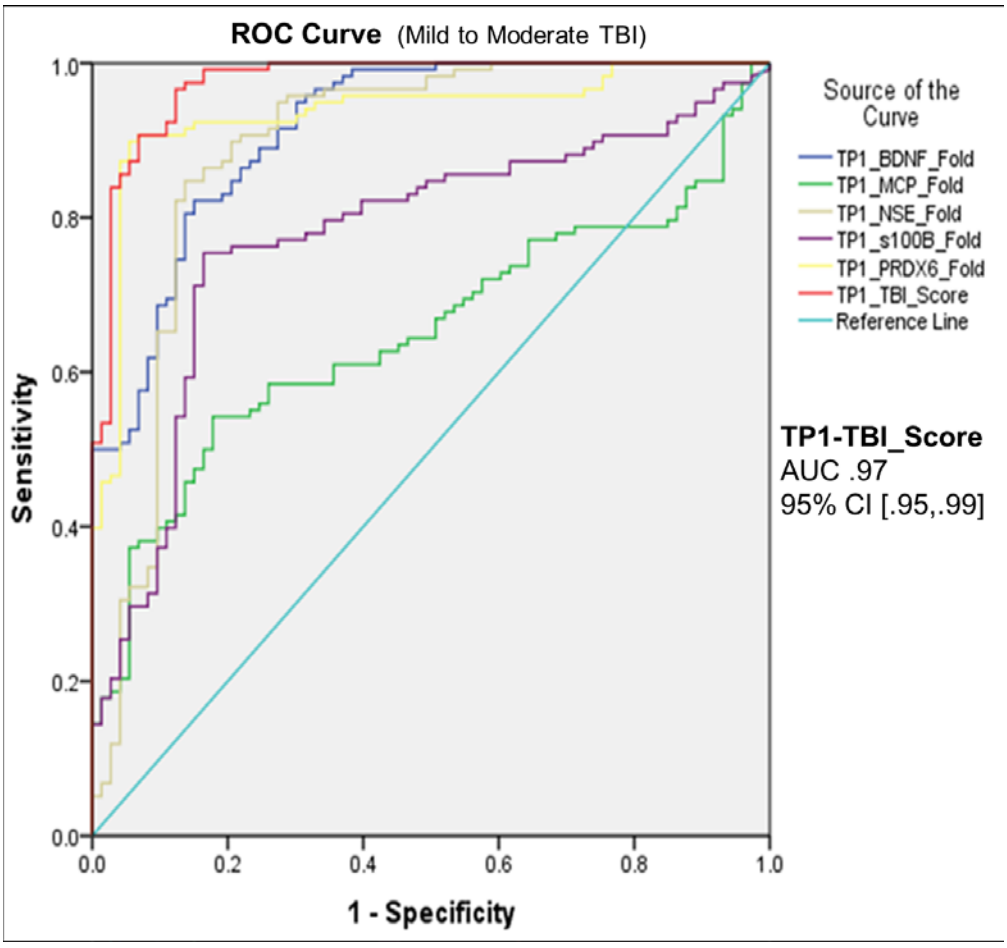

Figure 16. Individual receiver operating characteristics for Cohort 1 (mild-moderate TBI) at time point (TP) 1. The red line demonstrates the combined ROC curves of 5 TBI Biomarkers. Note the high sensitivity and specificity (AUC .97) when comparing individual biomarkers are combined into a TBI assessment score.

Relationship of current findings to previous findings:

BIOMARKERS IN MEDICINE

The use of circulating biomarkers has been invaluable in identifying and assessing a wide array of disease states. Conditions of the heart, kidney, pancreas, colon, ovaries, uterus and prostate are all examples of organs for which biomarkers have been developed and successfully used for both diagnosis and disease assessment [5]. Sampling for blood-borne biomarkers is minimally invasive, a better predictor of illness than self-reporting, and offers an objective and quantitative measure of disease processes [6,7]. Health care professionals rarely rely exclusively on biomarkers for a diagnosis, but their addition to the armamentarium of decision-making tools has been extremely beneficial for a variety of medical conditions.

Biomarkers can be viewed as having three roles: they function as diagnostics, prognostics, and predictors [8]. The diagnostic role pertains to their ability to aid in diagnosing a condition before it is detectable by overt signs or symptoms. A biomarker's prognostic ability refers to its ability to forecast the aggressiveness of a disease process, or how a specific medical condition will be affected by direct intervention [8]. Finally, the predictive aspect of a biomarker relates to its quantitative nature, which can be interpreted in the assessment of disease status and the response to care and treatment.

Biomarkers can also be broken down into three types: (1) molecular or biochemical, which are often genes or proteins; (2) physiological, which pertains to functional processes such as blood flow after a stroke; and (3) anatomical, which are related to structures and relationships between parts, such as that seen in the movement disorder resulting from specific neurological deficits in Huntington's disease [8]. The research undertaken here focused on biochemical biomarkers relevant to TBI.

The use of diagnostic biomarkers requires assays that are evaluated on the basis of their specificity, sensitivity, validity, and reliability. Specificity refers to the accuracy of a biomarker test in identifying a positive result that is an accurate reflection of the patient's true condition. A high degree of specificity results in a low rate of false positives. Sensitivity describes the likelihood that the patient has a positive result when the protein is present: thereby a high sensitivity biomarker assures sensitive detection. Validity of a biomarker is often described in two terms. Clinical validity pertains to a biomarker's ability to accurately predict clinical outcome. Assay validity is the confirmation that the assay measures what it reports to measure. One example of a test to confirm validity would be the demonstration of an expected finding using an independent measure. For example, an antibody's specificity for a protein of interest in an immunosorbent electrochemiluminescent assay (IEA) could be confirmed by immunoblotting. Reliability of a biomarker requires that its assay is consistently repeatable and accurate. Having addressed the characteristics and clinical use of biomarkers, our attention will turn to a brief consideration of the evolution of TBI biomarkers, specifically pertaining to development and shortcomings.

BIOMARKERS IN TBI: GAPS TO BE FILLED

It is well recognized that blood-borne biomarkers have the potential to be useful and important tools in clinical medicine relating to brain injury. An objective assessment of the TBI biomarker field over the last decade reveals that an extraordinarily large volume of work has been conducted at considerable cost to the taxpayer and private investor. Much of this work has involved the selection of likely candidates, chosen based on deductive reasoning. If the protein was known to be brain-specific and was shown to produce a distinct peptide signature when digested with proteases known to be activated in TBI, then that protein and its fragments were

pursued as candidate TBI biomarkers. Table 11 presents a list of proteins widely recognized as potential biomarkers for TBI. Unfortunately, none of these biomarkers when used independently has proven useful in the diagnosis of mild TBI.

Table 11. Summary of the TBI biomarker literature for commonly cited markers

Known TBI Markers	**Reported findings and references**
GFAP	Increased levels are seen after TBI [9-12]. Elevated levels are predictive of poorer outcome and correlate with Glasgow Coma Scale and mortality. Good sensitivity (85%) but poor specificity (52%) for predicting poor outcome at 6 months [9].
S100b	Serum and CSF levels increase following TBI, and were shown to predict outcome, differentiates mild and severe injury and correlates with CT scans [9,10,13-20]. S100b is a sensitive marker for TBI (95%) but with low specificity (31%) and not always specific to brain injury [9,13,21-24].
NSE	Shown to be a marker of brain injury severity, and levels have been correlated with poor outcome in TBI patients [14,25,26]. Good sensitivity (80%) but poor specificity (55%) [27].
CKBB	One of the first TBI biomarkers studied. 2009 study in military personnel with suspected mild TBI serving in Iraq demonstrated poor sensitivity (11%) and good specificity (97%) [26,28-37].
UCH-L1	Serum levels increase with TBI and levels are associated with severity of injury and CT scan results [38,39]. Also shown as a predictor of in-hospital mortality and strongly predicted death within 6 months. Not significantly elevated in mild TBI [40].
MBP	Levels show some correlation with outcome but lack specificity [41,42].
α II-spectrin proteolysis products	Shown to correlate with severity of injury [43-45]. Another study correlated levels with severity and outcome 6 months post-injury in severe TBI [46]. Higher levels are found in patients with poor outcome [47].
BDNF	A body of evidence is forming that points to dysregulation of BDNF following TBI and post-traumatic stress disorders [48-52].

The leader in TBI biomarker research, Banyan Biomarkers, Inc., has received more than $75 million in grants and contracts over the last ten years to support its discovery efforts [53]. The company has made significant progress in developing and clinically validating many enzyme-linked immunosorbent assays (ELISAs) for TBI. While Banyan's scientists have created an extensive portfolio of intellectual property and a large pipeline of potential biomarkers, the fact

remains that neither Banyan nor any other research enterprise has been successful in developing biomarkers for assessing anything other than the more severe forms of TBI: that is, conditions where the obviousness of the injury diminishes the need for biomarker assessment. The two lead candidates under development for Food and Drug Administration approval, ubiquitin carboxy-terminal hydrolase - L1 (UCH-L1) and a proteolytic fragment of glial fibrillary acidic protein (GFAP), have proven ineffective at identifying milder forms of TBI. There is no compelling evidence that any other Banyan candidates have any better potential [39,54-56]. Accordingly, a new approach to biomarker discovery is needed.

HOW THIS RESEARCH FILLS GAPS IN TBI SCIENCE

This research introduces a new strategy for the discovery of novel TBI biomarkers and demonstrates how a panel of biomarkers can be used to formulate an assessment score sensitive for mild TBI. The research is based upon the proposal that the humoral immune response to brain injury can serve as a pathway for the discovery of TBI biomarkers. The underlying hypothesis for this investigation is that brain-specific autoantibodies can be used to identify proteins that will serve as circulating biomarkers for the assessment of mild TBI. The objectives for this project, therefore, were to identify and investigate brain proteins that are targeted by autoimmune recognition in response to TBI. The ultimate goal for this work was to utilize this approach of autoimmune profiling to discover novel biomarkers useful in the diagnosis and assessment of TBI.

RELATION OF PRESENTED RESEARCH TO TBI FIELD

An important challenge facing discovery efforts for novel TBI biomarker proteins is the need for a discovery approach that does not rely upon an *a priori* search paradigm. This

dissertation project was designed to meet this challenge. The research presented here has added to the field of TBI biomarkers with two major outcomes. First, it was demonstrated that novel biomarkers for TBI can be discovered by autoimmune profiling. Importantly, the candidate biomarkers discovered by this approach are generally unique and have not been detected by conventional strategies for TBI biomarker discovery. The second major outcome from this dissertation project is the demonstration that a profile of biomarker responses can be used to formulate a diagnostic score that is sensitive for the detection of mild TBI.

Finally, an additional important outcome of this research is the identification of specific TBI-induced autoantibodies. The presence of these molecules introduces new mechanisms through which the immune system may contribute to the long-term negative consequences of TBI. The concept of centrally directed autoantibodies mediating long-term neuropathology is not without precedent, especially in the case of multiple sclerosis (MS) where autoimmune mechanisms are a well-recognized underpinning for the neuropathology of the disease [57]. It is proposed here that, similar to MS, autoantibodies generated in response to TBI may contribute to the long-term comorbidities associated with brain injury including seizures, epilepsy

Effect of problems or obstacles on the results:

Efforts to develop analytical tools for neuronal pentraxin 1, PCLO, CRMP2, and mu-crystallin homolog were met with limited success and did not yield IEAs suitable for the analyses needed here. This was due to either the absence of appropriate antibodies, standard proteins or a lack of assay sensitivity. Fortunately, the assays that were developed proved highly successful in establishing the TBI assessment score developed here. As research materials become available and more protein assays come online there is an opportunity for the TBI Assessment score to increase its sensitivity.

Despite significant effort, human traumatically injured brain tissue from our Glasgow Scotland Brain Bank Biorepository collaborators did not become available in time to be included in this body of work. Therefore, no human brain issue underwent immunohistochemical analysis. Fortunately, two collaborative relationships were established with a research group from NIH and one from the Defence Research and Development Canada, National Defence, Canada. These ongoing studies provided invaluable human serum from two large TBI cohort studies. The availability of the human serum samples created the opportunity to immediately implement the translational research conducted in the first part of research project.

Limitations:

There are several other considerations that are relevant to the interpretation of the findings presented here and the diagnosis of TBI. First, to the best of our knowledge, none of the proteins under consideration as TBI biomarkers (both here and by other labs) are strictly unique to the central nervous system. Accordingly, the concept of a brain–specific response must be qualified when discussing TBI and should include the potential contribution of poly-trauma to a biomarker signature. Second, in view of the statistic that nearly 1/3 of all traumas are associated with alcohol consumption, it is important to recognize the impact that alcohol has on the diagnosis of TBI. Alcohol intoxication compromises the value of neuropsychiatric testing normally performed as part of the TBI assessment. The effect, if any, of alcohol consumption on blood levels of specific biomarkers has yet to be determined. Third, demographic differences including age, gender and genetic background as well as concurrent and past medical conditions, (especially a history of recurrent head injury) may all influence measures of TBI biomarkers. Fourth, different mechanisms of neurological injury may result in different biomarker responses. This may be the case for TBI due to its highly varied injury types that include penetrating injury,

acceleration/deceleration injury, falls, and head striking object/object striking head. Fifth, due to the heterogeneous nature of the human condition, longitudinal studies are required for the most meaningful assessment of biomarker responses in TBI. In this regard, military service members offer a unique study population where pre-deployment blood samples are routinely collected and thus are available for evaluating the entire spectrum of deployment experiences including the stress of arriving in theater, combat operations and post-deployment reintegration. In the event of a TBI, sequential sampling provides an excellent opportunity for following biomarker responses post-injury. Sixth, validation of the biomarkers discovered here was hindered by the lack of commercially available antibodies and protein standards. Accordingly, development of specific monoclonal antibodies and protein standards is necessary to advance the development of a number of our TBI biomarker candidates. Seventh, it is important to pursue the role that disruption in the BBB has in the autoimmune response to TBI. Specifically, it is important to determine if disruption of the BBB is necessary for the generation of autoantibodies following TBI and further, the extent to which pharmacological or inflammatory disruptions of the BBB may produce autoimmune responses similar to those of TBI. Finally, this work brings forward an intriguing concept concerning the role brain-centric autoantibodies have in the long-term consequences of TBI. Autoimmune-based neuropathologies could be evaluated in models examining the effects of aggressive immunosuppression on the recovery of behavioral and motor functions following TBI.

Conclusion:

This research has two major outcomes which are medically relevant in the mTBI research. First, it demonstrates that autoimmune profiling can be used to identify novel

biomarkers for TBI. Second, this investigation demonstrates for the first time that a profile of biomarker responses can form the basis for a diagnostic assessment score that is sensitive for the detection of mTBI and can be standardized across clinical settings.

The high rate of head trauma in deployed military personnel and in civilians involved in automobile accidents and in contact sports is well recognized. The extent to which these head traumas result in mild brain injury, however, cannot be determined due to the lack of suitable diagnostic tools, including biomarkers. At present, there is no effective way to assess mild traumatic brain injury (TBI) and the risks it brings for underlying neuropathology and second injury syndrome. For more than a decade, traditional approaches in biomarker research have failed to identify a means for diagnosing mild TBI. Much of this work has focused on single proteins thought to be relevant to TBI but subsequently shown to be ineffective for the diagnosis of mild TBI. It is now clear that new approaches to the discovery of biomarkers and their applications in diagnosis are needed. The goals of this research were to identify novel brain proteins targeted by TBI-induced autoantibodies and to determine if these proteins contribute to a circulating biomarker signature for TBI. We reasoned that the humoral immune response to brain injury may serve as a pathway for the discovery of novel biomarker proteins for TBI.

It was shown that the plasma profiles of these novel biomarkers, when considered in conjunction with the profiles of other established biomarkers, could be interpreted to create an assessment score that identifies mild TBI. Specifically, it was demonstrated how fold changes in plasma levels of a panel of biomarker proteins can be formulated to produce a TBI Assessment Score that identifies mild TBI in humans. This score offers a long-sought solution to the need for a sensitive and objective tool for diagnosing mild TBI in adult patients. Because the TBI

Assessment Score is based upon definitive measures of circulating biomarkers, it is an objective assessment that is easily standardized across clinical settings.

In summary, this research has two major outcomes. First, it has demonstrated that autoimmune profiling can be used to identify novel biomarkers for TBI. Importantly, the candidate biomarkers identified by this approach are unique and have not been detected by conventional strategies for TBI biomarker discovery. The second major outcome from this investigation is the demonstration that a profile of biomarker responses can be used to formulate a diagnostic score that is sensitive for the detection of mild TBI. It is proposed that this concept of a multivariate TBI Assessment Score will be refined with more sensitive and specific biomarkers that will effectively define the entire spectrum of TBI. Finally, an additional important aspect of this research is the identification of specific TBI-induced autoantibodies. The presence of these molecules introduces new mechanisms through which the immune system may contribute to the long-term negative consequences of TBI. Understanding the details of the autoimmune response to TBI will guide treatments designed to regulate the immune response to TBI, and thereby optimize neuroregeneration and restoration of functional circuits.

Significance of Study or Project Results to Military Nursing

Despite substantial scientific effort, no biomarkers have been established for the diagnosis of mild / moderate TBI or assessing its response to therapy. Accordingly, optimal treatment strategies cannot be determined. Considering the significant number of warriors injured, it is imperative that part of our military nursing research focus on enhancing nursing assessment of TBI to provide the best possible care tailored to the specific needs of each patient. In addition, this assessment will contribute to the clinician's determination on the return-to-duty status.

Nurses are among the first healthcare professionals to triage our warriors. In the fall of 2010 MG James K. Gilman Commanding General, U.S Army Medical Research and Materiel Command USAMRMC presented *Army initiatives to address TBI, Post traumatic Stress Disorder, and Suicide*[96] to the Army Science Conference. General Gilman listed biomarkers as one of the research solutions to address the medical capability needed to aid in head injury assessment. The knowledge gained through this work will directly assist in the acute assessment of head injury victims. This knowledge, coupled with a more complete understanding of the pathologies responsible for the consequences of traumatic brain injury, will lead to improvements in the nursing algorithms currently in use for diagnosing, treating and assessing response to therapy following TBI.

The National Research Council recognizes "biomarkers of effect" as those that reflect a condition or disease state through measurable variations in bodily fluids [97]. To be effective, these markers must meet the criteria of high specificity and sensitivity and ease of detection. Also important are ease of sample collection and sufficient sample volume to allow for repeated testing. The fundamental limitation of all current TBI biomarkers is clear: when studied

individually, none of the established biomarkers for TBI have sufficient specificity or sensitivity for accurate diagnosis of mild TBI. This research presented a straightforward approach that effectively utilizes the strengths of currently available biomarkers in combination with newly discovered biomarkers to develop a diagnostic tool that is sensitive to mild TBI.

Unlike the conventional approach which is based upon the biased *a priori* selection of each specific protein to be studied, autoimmune profiling investigates the immune response to identify proteins that are directly involved in TBI. In this manner, autoimmune profiling is unbiased and evaluates the entire proteome in the search for candidates that are functionally involved in the injury as judged by the expression of TBI-induced autoantibodies.

Use of this innovative approach has led to the discovery of a panel of completely novel candidate biomarkers for brain injury. An additional outcome of potential clinical relevance is the possibility that the autoantibodies involved may themselves constitute a diagnostic signature for TBI. This latter outcome points to the potential involvement of the humoral immune system in the long-term pathology of TBI.

In summary, this research introduces a new approach for the discovery of TBI biomarker proteins. This approach has resulted in the identification of a panel of novel biomarker candidates. The findings presented here demonstrate how patterns in biomarker responses for both novel and established biomarker proteins can be formulated to create a single, objective, multivariate TBI Assessment Score that is sensitive to mild TBI. Ultimately, this work has the potential to directly affect the advanced practice nurse’s assessment of TBI patients

Changes in Clinical Practice, Leadership, Management, Education, Policy, and/or Military Doctrine that Resulted from Study or Project

None to date

References Cited - Bibliography

1. Tabachnick BG, Fidell, L.S. . *Using Multivariate Statistics, 5/E.* Pearson; 2006.
2. Marshall LF, Marshall SB, Klauber MR, et al. The diagnosis of head injury requires a classification based on computed axial tomography. *J Neurotrauma.* Mar 1992;9 Suppl 1:S287-292.
3. Wilson JT, Pettigrew LE, Teasdale GM. Structured interviews for the Glasgow Outcome Scale and the extended Glasgow Outcome Scale: guidelines for their use. *J Neurotrauma.* Aug 1998;15(8):573-585.
4. Teasdale G, Jennett B. Assessment and prognosis of coma after head injury. *Acta Neurochir (Wien).* 1976;34(1-4):45-55.
5. Korfias S, Papadimitriou A, Stranjalis G, et al. Serum biochemical markers of brain injury. *Mini reviews in medicinal chemistry.* Feb 2009;9(2):227-234.
6. Goldman N. Future Directions in Integration of Biological and Social Theories. Paper presented at: 5th Annual Workshop on Biomarker Collection in Population-Based Health Research2007; Chicago, IL.
7. PRB. Use of Biomarkers in Predicting Health and Mortality. *Today's Research on Aging - Program and Policy Implications.* 2008(14). www.prb.org.
8. RAN. Biomarkers in Cancer - An introductory Guide to Advocates. 2010. http://researchadvocacy.org/images/uploads/downloads/BiomarkerinCancer_WebDownloadVersion.pdf.
9. Vos PE, Lamers KJ, Hendriks JC, et al. Glial and neuronal proteins in serum predict outcome after severe traumatic brain injury. *Neurology.* Apr 27 2004;62(8):1303-1310.
10. Pelinka LE, Kroepfl A, Schmidhammer R, et al. Glial fibrillary acidic protein in serum after traumatic brain injury and multiple trauma. *The Journal of trauma.* Nov 2004;57(5):1006-1012.
11. Nylen K, Ost M, Csajbok LZ, et al. Increased serum-GFAP in patients with severe traumatic brain injury is related to outcome. *J Neurol Sci.* Jan 15 2006;240(1-2):85-91.
12. Wiesmann M, Steinmeier E, Magerkurth O, Linn J, Gottmann D, Missler U. Outcome prediction in traumatic brain injury: comparison of neurological status, CT findings, and blood levels of S100B and GFAP. *Acta neurologica Scandinavica.* Mar 2010;121(3):178-185.
13. Biberthaler P, Linsenmeier U, Pfeifer KJ, et al. Serum S-100B concentration provides additional information fot the indication of computed tomography in patients after minor head injury: a prospective multicenter study. *Shock.* May 2006;25(5):446-453.
14. Herrmann M, Curio N, Jost S, Wunderlich MT, Synowitz H, Wallesch CW. Protein S-100B and neuron specific enolase as early neurobiochemical markers of the severity of traumatic brain injury. *Restor Neurol Neurosci.* 1999;14(2-3):109-114.
15. Pelinka LE, Kroepfl A, Leixnering M, Buchinger W, Raabe A, Redl H. GFAP versus S100B in serum after traumatic brain injury: relationship to brain damage and outcome. *J Neurotrauma.* Nov 2004;21(11):1553-1561.
16. Woertgen C, Rothoerl RD, Holzschuh M, Metz C, Brawanski A. Comparison of serial S-100 and NSE serum measurements after severe head injury. *Acta Neurochir (Wien).* 1997;139(12):1161-1164; discussion 1165.

17. McKeating EG, Andrews PJ, Mascia L. Relationship of neuron specific enolase and protein S-100 concentrations in systemic and jugular venous serum to injury severity and outcome after traumatic brain injury. *Acta Neurochir Suppl.* 1998;71:117-119.
18. Raabe A, Grolms C, Keller M, Dohnert J, Sorge O, Seifert V. Correlation of computed tomography findings and serum brain damage markers following severe head injury. *Acta Neurochir (Wien).* 1998;140(8):787-791; discussion 791-782.
19. Rothoerl RD, Woertgen C, Holzschuh M, Metz C, Brawanski A. S-100 serum levels after minor and major head injury. *The Journal of trauma.* Oct 1998;45(4):765-767.
20. Zurek J, Fedora M. The usefulness of S100B, NSE, GFAP, NF-H, secretagogin and Hsp70 as a predictive biomarker of outcome in children with traumatic brain injury. *Acta Neurochir (Wien).* Jan 2012;154(1):93-103; .
21. Herrmann M, Jost S, Kutz S, et al. Temporal profile of release of neurobiochemical markers of brain damage after traumatic brain injury is associated with intracranial pathology as demonstrated in cranial computerized tomography. *J Neurotrauma.* Feb 2000;17(2):113-122.
22. Rothoerl RD, Woertgen C. High serum S100B levels for trauma patients without head injuries. *Neurosurgery.* Dec 2001;49(6):1490-1491; author reply 1492-1493.
23. Anderson RE, Hansson LO, Nilsson O, Dijlai-Merzoug R, Settergren G. High serum S100B levels for trauma patients without head injuries. *Neurosurgery.* Jun 2001;48(6):1255-1258; discussion 1258-1260.
24. Romner B, Ingebrigtsen T. High serum S100B levels for trauma patients without head injuries. *Neurosurgery.* Dec 2001;49(6):1490; author reply 1492-1493.
25. Ross SA, Cunningham RT, Johnston CF, Rowlands BJ. Neuron-specific enolase as an aid to outcome prediction in head injury. *British journal of neurosurgery.* Oct 1996;10(5):471-476.
26. Skogseid IM, Nordby HK, Urdal P, Paus E, Lilleaas F. Increased serum creatine kinase BB and neuron specific enolase following head injury indicates brain damage. *Acta Neurochir (Wien).* 1992;115(3-4):106-111.
27. Pelinka LE, Hertz H, Mauritz W, et al. Nonspecific increase of systemic neuron-specific enolase after trauma: clinical and experimental findings. *Shock.* Aug 2005;24(2):119-123.
28. Bazarian JJ, Beck C, Blyth B, von Ahsen N, Hasselblatt M. Impact of creatine kinase correction on the predictive value of S-100B after mild traumatic brain injury. *Restor Neurol Neurosci.* 2006;24(3):163-172.
29. Carr ME, Jr., Masullo LN, Brown JK, Lewis PC. Creatine kinase BB isoenzyme blood levels in trauma patients with suspected mild traumatic brain injury. *Military medicine.* Jun 2009;174(6):622-625.
30. Graham MR, Myers T, Evans P, et al. Direct hits to the head during amateur boxing is associated with a rise in serum biomarkers for brain injury. *International journal of immunopathology and pharmacology.* Jan-Mar 2011;24(1):119-125.
31. Hergenroeder GW, Redell JB, Moore AN, Dash PK. Biomarkers in the clinical diagnosis and management of traumatic brain injury. *Mol Diagn Ther.* 2008;12(6):345-358.
32. Ingebrigtsen T, Romner B. Biochemical serum markers for brain damage: a short review with emphasis on clinical utility in mild head injury. *Restor Neurol Neurosci.* 2003;21(3-4):171-176.
33. Karkela J, Bock E, Kaukinen S. CSF and serum brain-specific creatine kinase isoenzyme (CK-BB), neuron-specific enolase (NSE) and neural cell adhesion molecule (NCAM) as

prognostic markers for hypoxic brain injury after cardiac arrest in man. *J Neurol Sci.* May 1993;116(1):100-109.
34. Kato K, Suzuki F, Shimizu A, Shinohara H, Semba R. Highly Sensitive Immunoassay for Rat Brain-Type Creatine Kinase: Determination in Isolated Purkinje Cells. *Journal of Neurochemistry.* 1986;46(6):1783-1788.
35. Phillips JP, Jones HM, Hitchcock R, Adama N, Thompson RJ. Radioimmunoassay of serum creatine kinase BB as index of brain damage after head injury. *British medical journal.* Sep 20 1980;281(6243):777-779.
36. Thompson RJ, Graham JG, McQueen IN, Kynoch PA, Brown KW. Radioimmunoassay of brain-type creatine kinase-BB isoenzyme in human tissues and in serum of patients with neurological disorders. *J Neurol Sci.* Aug 1980;47(2):241-254.
37. Willson VJ, Jones HM, Morgan PM, Thompson RJ. A two-site immunoradiometric assay for the BB isoenzyme of human creatine kinase. *Biochemical Society transactions.* Oct 1980;8(5):621-622.
38. Mondello S, Muller U, Jeromin A, Streeter J, Hayes RL, Wang KK. Blood-based diagnostics of traumatic brain injuries. *Expert review of molecular diagnostics.* Jan 2011;11(1):65-78.
39. Papa L, Akinyi L, Liu MC, et al. Ubiquitin C-terminal hydrolase is a novel biomarker in humans for severe traumatic brain injury. *Critical care medicine.* Jan 2010;38(1):138-144.
40. Berger RP, Hayes RL, Richichi R, Beers SR, Wang KK. Serum concentrations of ubiquitin C-terminal hydrolase-L1 and alphaII-spectrin breakdown product 145 kDa correlate with outcome after pediatric TBI. *J Neurotrauma.* Jan 1 2012;29(1):162-167.
41. Berger RP, Adelson PD, Pierce MC, Dulani T, Cassidy LD, Kochanek PM. Serum neuron-specific enolase, S100B, and myelin basic protein concentrations after inflicted and noninflicted traumatic brain injury in children. *Journal of neurosurgery.* Jul 2005;103(1 Suppl):61-68.
42. Thomas DG, Rabow L, Teasdale G. Serum myelin basic protein, clinical responsiveness, and outcome of severe head injury. *Acta neurochirurgica. Supplementum.* 1979;28(1):93-95.
43. Wang KK, Ottens AK, Liu MC, et al. Proteomic identification of biomarkers of traumatic brain injury. *Expert Rev Proteomics.* Aug 2005;2(4):603-614.
44. Warren MW, Kobeissy FH, Liu MC, Hayes RL, Gold MS, Wang KK. Concurrent calpain and caspase-3 mediated proteolysis of alpha II-spectrin and tau in rat brain after methamphetamine exposure: a similar profile to traumatic brain injury. *Life Sci.* Dec 5 2005;78(3):301-309.
45. Warren MW, Zheng W, Kobeissy FH, et al. Calpain- and caspase-mediated alphaII-spectrin and tau proteolysis in rat cerebrocortical neuronal cultures after ecstasy or methamphetamine exposure. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP).* Aug 2007;10(4):479-489.
46. Pineda JA, Lewis SB, Valadka AB, et al. Clinical significance of alphaII-spectrin breakdown products in cerebrospinal fluid after severe traumatic brain injury. *J Neurotrauma.* Feb 2007;24(2):354-366.

47. Cardali S, Maugeri R. Detection of alphaII-spectrin and breakdown products in humans after severe traumatic brain injury. *Journal of neurosurgical sciences.* Jun 2006;50(2):25-31.
48. Bejot Y, Mossiat C, Giroud M, Prigent-Tessier A, Marie C. Circulating and brain BDNF levels in stroke rats. Relevance to clinical studies. *PLoS ONE.* 2011;6(12):e29405.
49. Berzi A, Ayata CK, Cavalcante P, et al. BDNF and its receptors in human myasthenic thymus: implications for cell fate in thymic pathology. *J Neuroimmunol.* Jul 15 2008;197(2):128-139.
50. Ring RH, Alder J, Fennell M, Kouranova E, Black IB, Thakker-Varia S. Transcriptional profiling of brain-derived-neurotrophic factor-induced neuronal plasticity: a novel role for nociceptin in hippocampal neurite outgrowth. *Journal of neurobiology.* Mar 2006;66(4):361-377.
51. Sharma HS, Zimmermann-Meinzingen S, Johanson CE. Cerebrolysin reduces blood-cerebrospinal fluid barrier permeability change, brain pathology, and functional deficits following traumatic brain injury in the rat. *Ann N Y Acad Sci.* Jun 2010;1199:125-137.
52. Kaplan GB, Vasterling JJ, Vedak PC. Brain-derived neurotrophic factor in traumatic brain injury, post-traumatic stress disorder, and their comorbid conditions: role in pathogenesis and treatment. *Behavioural pharmacology.* Sep 2010;21(5-6):427-437.
53. TechConnect U. Banyan Biomarkers, Inc. Monitoring Traumatic Brain Injury. In: Florida Uo, ed. *UF TechConnect*. Gainsville, FL: UF Office of Technology Licensing; 2011.
54. Czeiter E, Mondello S, Kovacs N, et al. Brain injury biomarkers may improve the predictive power of the IMPACT outcome calculator. *J Neurotrauma.* Jun 10 2012;29(9):1770-1778.
55. Mondello S, Linnet A, Buki A, et al. Clinical utility of serum levels of ubiquitin C-terminal hydrolase as a biomarker for severe traumatic brain injury. *Neurosurgery.* Mar 2012;70(3):666-675.
56. Mondello S, Papa L, Buki A, et al. Neuronal and glial markers are differently associated with computed tomography findings and outcome in patients with severe traumatic brain injury: a case control study. *Critical care (London, England).* 2011;15(3):R156.
57. Kasper LH, Shoemaker J. Multiple sclerosis immunology: The healthy immune system vs the MS immune system. *Neurology.* Jan 5 2010;74 Suppl 1:S2-8.
58. Neumann CA, Krause DS, Carman CV, et al. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. *Nature.* Jul 31 2003;424(6948):561-565.
59. Sorokina EM, Feinstein SI, Zhou S, Fisher AB. Intracellular targeting of peroxiredoxin 6 to lysosomal organelles requires MAPK activity and binding to 14-3-3epsilon. *American journal of physiology. Cell physiology.* Jun 2011;300(6):C1430-1441.
60. Tulsawani R, Kelly LS, Fatma N, et al. Neuroprotective effect of peroxiredoxin 6 against hypoxia-induced retinal ganglion cell damage. *BMC neuroscience.* 2010;11:125.
61. Power JH, Asad S, Chataway TK, et al. Peroxiredoxin 6 in human brain: molecular forms, cellular distribution and association with Alzheimer's disease pathology. *Acta Neuropathol.* Jun 2008;115(6):611-622.
62. Woertgen C, Rothoerl RD, Wiesmann M, Missler U, Brawanski A. Glial and neuronal serum markers after controlled cortical impact injury in the rat. *Acta Neurochir Suppl.* 2002;81:205-207.

63. Woertgen C, Rothoerl RD, Brawanski A. Neuron-specific enolase serum levels after controlled cortical impact injury in the rat. *J Neurotrauma.* May 2001;18(5):569-573.
64. Bohmer AE, Oses JP, Schmidt AP, et al. Neuron-specific enolase, S100B, and glial fibrillary acidic protein levels as outcome predictors in patients with severe traumatic brain injury. *Neurosurgery.* Jun 2011;68(6):1624-1630; discussion 1630-1621.
65. Dogan RN, Karpus WJ. Chemokines and chemokine receptors in autoimmune encephalomyelitis as a model for central nervous system inflammatory disease regulation. *Frontiers in bioscience : a journal and virtual library.* May 1 2004;9:1500-1505.
66. Mahad D, Callahan MK, Williams KA, et al. Modulating CCR2 and CCL2 at the blood-brain barrier: relevance for multiple sclerosis pathogenesis. *Brain.* Jan 2006;129(Pt 1):212-223.
67. Mahad DJ, Ransohoff RM. The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). *Seminars in immunology.* Feb 2003;15(1):23-32.
68. Yang H. Structure, Expression, and Function of ICAM-5. *Comparative and functional genomics.* 2012;2012:368938.
69. Maruya SI, Myers JN, Weber RS, Rosenthal DI, Lotan R, El-Naggar AK. ICAM-5 (telencephalin) gene expression in head and neck squamous carcinoma tumorigenesis and perineural invasion! *Oral oncology.* Jul 2005;41(6):580-588.
70. Tse MC, Lane C, Mott K, Onlamoon N, Hsiao HM, Perng GC. ICAM-5 modulates cytokine/chemokine production in the CNS during the course of herpes simplex virus type 1 infection. *J Neuroimmunol.* Aug 18 2009;213(1-2):12-19.
71. Gahmberg CG, Tian L, Ning L, Nyman-Huttunen H. ICAM-5--a novel two-facetted adhesion molecule in the mammalian brain. *Immunology letters.* May 15 2008;117(2):131-135.
72. Ning L, Tian L, Smirnov S, et al. Interactions between ICAM-5 and beta1 integrins regulate neuronal synapse formation. *Journal of cell science.* Jan 1 2013;126(Pt 1):77-89.
73. Tian L, Nyman H, Kilgannon P, et al. Intercellular adhesion molecule-5 induces dendritic outgrowth by homophilic adhesion. *The Journal of cell biology.* Jul 10 2000;150(1):243-252.
74. Kilgannon P, Turner T, Meyer J, Wisdom W, Gallatin WM. Mapping of the ICAM-5 (telencephalin) gene, a neuronal member of the ICAM family, to a location between ICAM-1 and ICAM-3 on human chromosome 19p13.2. *Genomics.* Dec 1 1998;54(2):328-330.
75. Camins A, Verdaguer E, Folch J, Canudas AM, Pallas M. The role of CDK5/P25 formation/inhibition in neurodegeneration. *Drug news & perspectives.* Oct 2006;19(8):453-460.
76. Cheung ZH, Gong K, Ip NY. Cyclin-dependent kinase 5 supports neuronal survival through phosphorylation of Bcl-2. *J Neurosci.* May 7 2008;28(19):4872-4877.
77. Cheung ZH, Ip NY. Cdk5 in Dendrite and Synapse Development: Emerging Role as a Modulator of Receptor Tyrosine Kinase Signaling
In: Ip NY, Tsai L-H, eds. *Cyclin Dependent Kinase 5 (Cdk5)*
Springer US; 2008:51-68.

78. Crews L, Ruf R, Patrick C, et al. Phosphorylation of collapsin response mediator protein-2 disrupts neuronal maturation in a model of adult neurogenesis: Implications for neurodegenerative disorders. *Molecular Neurodegeneration.* 2011;6(1):67.
79. Demange L, Abdellah FN, Lozach O, et al. Potent inhibitors of CDK5 derived from roscovitine: Synthesis, biological evaluation and molecular modelling. *Bioorganic & medicinal chemistry letters.* Jan 1 2013;23(1):125-131.
80. Dhariwala FA, Rajadhyaksha MS. An unusual member of the Cdk family: Cdk5. *Cellular and molecular neurobiology.* May 2008;28(3):351-369.
81. Dhavan R, Tsai LH. A decade of CDK5. *Nature reviews. Molecular cell biology.* Oct 2001;2(10):749-759.
82. Goshima Y, Sasaki Y, Uchida Y, Yamashita N, Nakamura F. CRMP Family Protein: Novel Targets for Cdk5 That Regulates Axon Guidance, Synapse Maturation, and Cell Migration

Cyclin Dependent Kinase 5 (Cdk5). In: Ip NY, Tsai L-H, eds: Springer US; 2008:9-24.
83. Hensley K, Venkova K, Christov A, Gunning W, Park J. Collapsin Response Mediator Protein-2: An Emerging Pathologic Feature and Therapeutic Target for Neurodisease Indications. *Molecular Neurobiology.* 2011;43(3):180-191.
84. Lopes JP, Agostinho P. Cdk5: multitasking between physiological and pathological conditions. *Progress in neurobiology.* Jun 2011;94(1):49-63.
85. Mazanetz MP, Fischer PM. Untangling tau hyperphosphorylation in drug design for neurodegenerative diseases. *Nature reviews. Drug discovery.* Jun 2007;6(6):464-479.
86. Daly C, Rollins BJ. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation (New York, N.Y. : 1994).* Jun 2003;10(3-4):247-257.
87. Marchi N, Bazarian JJ, Puvenna V, et al. Consequences of repeated blood-brain barrier disruption in football players. *PLoS ONE.* 2013;8(3):e56805.
88. Kanwar JR, Sriramoju, B., Kanwar, R.K. Recent Advances in the Treatment of Neurological Autoimmune Disorders. In: Huang FP, ed. *Recent Advances in the Treatment of Neurological Autoimmune Disorders, Autoimmune Disorders - Current Concepts and Advances from Bedside to Mechanistic Insights*: InTech; 2011.
89. Davidson A, Diamond B. Autoimmune Diseases. *New England Journal of Medicine.* 2001;345(5):340-350.
90. Nagele E, Han M, Demarshall C, Belinka B, Nagele R. Diagnosis of Alzheimer's disease based on disease-specific autoantibody profiles in human sera. *PLoS ONE.* 2011;6(8):e23112.
91. Han M, Nagele E, DeMarshall C, Acharya N, Nagele R. Diagnosis of Parkinson's disease based on disease-specific autoantibody profiles in human sera. *PLoS ONE.* 2012;7(2):e32383.
92. Bouras C, Riederer BM, Kovari E, Hof PR, Giannakopoulos P. Humoral immunity in brain aging and Alzheimer's disease. *Brain Res Brain Res Rev.* Jun 2005;48(3):477-487.
93. Thompson RJ. Clinical applications of measurements of human brain-specific proteins. *Biochemical Society transactions.* Oct 1980;8(5):494-496.
94. Jun BH, Kang H, Lee YS, Jeong DH. Fluorescence-based multiplex protein detection using optically encoded microbeads. *Molecules (Basel, Switzerland).* 2012;17(3):2474-2490.

95. Burbelo PD, Hirai H, Leahy H, et al. A New Luminescence Assay for Autoantibodies to Mammalian Cell–Prepared Insulinoma-Associated Protein 2. *Diabetes Care.* September 2008 2008;31(9):1824-1826.
96. Gilman JK. Army Initiatives to Address TBI, PTSD, and Suicide. Paper presented at: 27th Army Science Conference, Translational Science and Technology, Enabling Full Spectrum Operations; November 30, 2010, 2010; Orlando FL.
97. NRC. Biological markers in environmental health research. Committee on Biological Markers of the National Research Council. *Environ Health Perspect.* Oct 1987;74:3-9.

Summary of Dissemination

Type of Dissemination	Citation	Date and Source of Approval for Public Release
Publications	John E. Buonora Dissertation - Development of a Traumatic Brain Injury Assessment Score Using Novel Biomarkers DiscoveredThrough Autoimmune Profiling.	08August 2013, USUHS Graduate Education
Published Abstracts	**John E. Buonora** US Army Graduate Program in Anesthesia Nursing, JBSA-FSH, TX. Development of a Traumatic Brain Injury Assessment Score Using Novel Biomarkers Discovered Through Autoimmune Profiling. 3rd Annual Academy of Health Sciences Graduate School Research Day, 12/13, JBSA-FSH, TX The United States Army Medical Department Journal, Jan-Mar 2014	December 2013 AMEDDC&S

Podium Presentations	**John E. Buonora**[1] [1] US Army Graduate Program in Anesthesia Nursing, JBSA-FSH, TX. Development of a Traumatic Brain Injury Assessment Score Using Novel Biomarkers Discovered Through Autoimmune Profiling. AANA 2014 Nurse Anesthesia Annual Congress. 09/14, Orlando, FL	24June2014, AMEDD C&S
	John E. Buonora[1] [1] US Army Graduate Program in Anesthesia Nursing, JBSA-FSH, TX. Development of a Traumatic Brain Injury Assessment Score Using Novel Biomarkers Discovered Through Autoimmune Profiling. 3rd Annual Academy of Health Sciences Graduate School Research Day, 12/13, JBSA-FSH, TX	N/A
	John E. Buonora[1], Carol Moore[2], Ramon Diaz-Arrastia[2], Harvey Pollard[3], Gregory P. Mueller[3] [1] Program in Neuroscience, Departments of [2]Neurology, [3]Anatomy, Physiology and Genetics, USUHS,Bethesda, MD. Immune Response Biomarker Profiling for Human Traumatic Brain Injury. Center for Neuroscience and Regenerative Medicine Annual Meeting, 05/12, Bethesda, MD.	N/A

Poster Presentations	**John E. Buonora**[1] [1] US Army Graduate Program In Anesthesia Nursing, JBSA-FSH, TX. *Abstract* Development of a Traumatic Brain Injury Assessment Score Using Novel Biomarkers Discovered Through Autoimmune Profiling. AANA 2014 Nurse Anesthesia Annual Congress. 09/14, Orlando, FL	24June2014, AMEDD C&S
	John E. Buonora*[1], Michael Mousseau*[2], Lawrence Latour[3], Ramon Diaz-Arrastia[4], Harvey Pollard[2], Sandro B. Rizoli[5], Andrew J. Baker[6], Shawn G. Rhind[7], Gregory P. Mueller[2] [1]Neuroscience, USUHS; [2]Anatomy, Physiology and Genetics, USUHS; [3]NIH/NINDS; [4]Neurology, USUHS, [5]Dept. of Surgery & Critical Care Medicine, Sunnybrook Health Sciences Centre; [6]Brain Injury Laboratory, Cara Phelan Centre for Trauma Research Keenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael's Hospital, University of Toronto; [7]Defence Research & Development Canada, Toronto, Canada. Peroxiredoxin 6: A Novel Candidate for a TBI Biomarker Signature. *Abstract*, 31st Annual National Neurotrauma Symposium, 08/13, Nashville, TN.	08August 2013, USUHS
	John E. Buonora[1], Carol Moore[2], Ramon Diaz-Arrastia[2], Harvey Pollard[3], Gregory P. Mueller[3] [1] Program in Neuroscience, Departments of [2]Neurology, [3]Anatomy, Physiology and Genetics, USUHS,Bethesda, MD. Immune Response Biomarker Profiling for Human Traumatic Brain Injury. *Abstract*, Center for Neuroscience and Regenerative Medicine Annual Meeting, 05/12, Bethesda, MD.	N/A

Reportable Outcomes

Reportable Outcome	Detailed Description
Applied for Patent	None
Issued a Patent	None
Developed a cell line	None
Developed a tissue or serum repository	None
Developed a data registry	None

Recruitment and Retention Table

Recruitment and Retention Aspect	Number
Animals Projected in Grant Application	114
Animals Purchased	114
Model Development Animals	5
Animals Intervention Group / Control or Sham Group	88/16
Intervention Group / Control or Sham Group Animals With Complete Data	88/16
Intervention Group / Control or Sham Group Animals With Incomplete Data	0/0

Final Budget Report

HENRY M. JACKSON FOUNDATION
FOR THE ADVANCEMENT OF MILITARY MEDICINE
Advancing Military Medical Research

August 12, 2014

Jody Milam
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD. 20814

Dear Mr. Floyd:

The following documents are attached to facilitate the closeout of Grant No. **HT9404-12-1-TS13. HJF PTA #306141-63891.**

A. Federal Financial Report
B. Grantee's Release Statement
C. Grantee's Assignment of Refunds, Rebates, Credits and other amounts

If you have any questions regarding these closeout documents, I can be contacted at (240) 694-2155.

Sincerely,

Katly Suarez
Financial Analyst

6720-A Rockledge Drive, Suite 100 • Bethesda, Maryland 20817
Tel: 240-694-2000 • Fax: 240-694-3100 • hjf.org

FEDERAL FINANCIAL REPORT

(Follow form instructions)

1. Federal Agency and Organizational Element to Which Report is Submitted	2. Federal Grant or Other Identifying Number Assigned by Federal Agency (To report multiple grants, use FFR Attachment)	Page	of
USUHS	HT9404-12-1-TS13, (N12-P12)	1	1 pages

3. Recipient Organization (Name and complete address including Zip code)

THE HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDICINE, INC.
6720- A Rockledge Dr., Suite 100, Bethesda, MD 20817

4a. DUNS Number	4b. EIN	5. Recipient Account Number or Identifying Number (To report multiple grants, use FFR Attachment)	6. Report Type	7. Basis of Accounting
144676566	52-1317896	306141 - 63891	☐ Quarterly ☐ Semi-Annual ☐ Annual ☑ Final	☐ Cash ☑ Accrual

8. Project/Grant Period		9. Reporting Period End Date
From: (Month, Day, Year) 07/01/2012	To: (Month, Day, Year) 06/30/2014	(Month, Day, Year) 06/30/14

10. Transactions	Cumulative
(Use lines a-c for single or multiple grant reporting)	
Federal Cash (To report multiple grants, also use FFR Attachment):	
a. Cash Receipts	$52,473.18
b. Cash Disbursements	$52,473.18
c. Cash on Hand (line a minus b)	$0.00
(Use lines d-o for single grant reporting)	
Federal Expenditures and Unobligated Balance:	
d. Total Federal funds authorized	$58,861.00
e. Federal share of expenditures	$52,473.18
f. Federal share of unliquidated obligations	$0.00
g. Total Federal share (sum of lines e and f)	$52,473.18
h. Unobligated balance of Federal funds (line d minus g)	$6,387.82
Recipient Share:	
i. Total recipient share required	$0.00
j. Recipient share of expenditures	$0.00
k. Remaining recipient share to be provided (line i minus j)	$0.00
Program Income:	
l. Total Federal program income earned	$0.00
m. Program income expended in accordance with the deduction alternative	$0.00
n. Program income expended in accordance with the addition alternative	$0.00
o. Unexpended program income (line l minus line m or line n)	

	a. Type	b. Rate	c. Period Fro	Period To	d. Base	e. Amount Charged	f. Federal Share
11. Indirect Expense	FIXED	45.069%	7/1/2012	9/30/2012	13,424.86	6,050.45	6,050.45
	FIXED	51.91%	10/1/2012	9/30/2013	21,721.47	11,276.40	11,276.40
	FIXED	56.00%	10/1/2013	6/30/2014	0.00	0.00	0.00
				g. Totals:	35,146.33	17,326.85	17,326.85

12. *Remarks:*

13. Certification: **By signing this report, I certify that it is true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent information may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 218, Section 10**

a. Typed or Printed Name and Title of Authorized Certifying Official	c. Telephone (Area code, number and extension)
Laraine Peng **Billing Manager**	**240-694-2008**
	d. Email address lpeng@hjf.org
b. Signature of Authorized Certifying Official	e. Date Report Submitted (Month, Day, Year) 8/6/14
	14. Agency use only:

Standard Form 425
OMB Approval Number: 0348-0061
Expiration Date: 10/31/2011

According to the Paperwork Reduction Act, as amended, no persons are required to respond to a collection of information unless it displays a valid OMB Control Number. The

GRANTEE'S ASSIGNMENT OF REFUNDS, CREDITS AND OTHER AMOUNTS

Grant Number HT9404-12-1-TS13, (N12-P12)

Pursuant to the terms of Grant Number HT9404-12-1-TS13, (N12-P12) and in consideration of the reimbursement of costs and payment of fee, as provided in the said grant and any assignment thereunder, The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., 6720A Rockledge Drive, Suite 100, Bethesda, MD 20817 (hereinafter called the Grantee) does hereby:

1. Assign, transfer, set over and release to the UNITED STATES OF AMERICA (hereinafter called the Government), all right, title and interest to all refunds, rebates, credits or other amounts (including any interest thereon) arising out of the performance of the said grant, together with all the rights of action accrued or which may hereinafter accrue thereunder.

2. Agree to take whatever action may be necessary to effect prompt collection of all refunds, rebates, credits or other amounts (including any interest thereon) due or which may become due and to promptly forward to the Grant Officer checks (made payable to the Treasurer of the United States) for any proceeds so collected. The reasonable cost of any such action to effect collection shall constitute allowable costs when approved by the Grant Officer as stated in the said grant and may be applied to reduce any amounts otherwise payable to the Government under the terms hereof.

3. Agree to cooperate fully with the Government as to any claim or suit in connection with refunds, rebates, credits or other amounts due (including any interest thereon); to execute any protest, pleading, application, power of attorney or other papers in connection therewith; and to permit the Government to represent it at any hearing, trial or other proceeding arising out of such claim or suit.

IN WITNESS WHEREOF, this assignment has been executed this 4TH day of August, 2014.

Ed Blaustein

(Grantee)

Ed Blaustein
Director of Finance

CERTIFICATE

I, William E. Slade, certify that I am the Secretary (Official Title) of the corporation named as Grantee in the foregoing assignment; that Ed Blaustein, who signed the said assignment on behalf of the Grantee was then Director of Finance (Official Title) of said corporation; that said assignment was duly signed for and in behalf of said corporation by authority of its governing body and is within the scope of its corporate powers.

(CORPORATE SEAL)

W.E. Slade

GRANTEE'S RELEASE

Grant Number HT9404-12-1-TS13, (N12-P12)

Pursuant to the terms of Grant Number HT9404-12-1-TS13, (N12-P12) and in consideration of the sum of Fifty Two Thousand Four Hundred Seventy-Three dollars and 18/100 ($52,473.18) which has been or is to be paid under the said grant to: The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., 6720A Rockledge Drive, Suite 100, Bethesda, MD 20817 (hereinafter called the Grantee) or to its assignees, if any, the Grantee, upon payment of the said sum by the UNITED STATES OF AMERICA (hereinafter called the Government), does remise, release, and discharge the Government, its officers, agents, and employees, of and from all liabilities, obligations, claims, and demands whatsoever under or arising from the said grant except:

1. Specified claims in stated amounts or in estimated amounts where the amounts are not susceptible of exact statement by the Grantee, as follows:

A. Claims, together with reasonable expenses incidental thereto, based upon the liabilities of the Grantee to third parties arising out of the performance of the said grant, which are not known to the Grantee on the date of the execution of this release and of which the Grantee gives notice in writing to the Grant Officer within the period specified in the said grant.

B. Claims of reimbursement of cost (other than expenses of the Grantee by reason of its indemnification of the Government against patent liability), including reasonable expenses incidental thereto, incurred by the Grantee under the provisions of the said grant relating patents.

The Grantee agrees, in connection with patent matters and with claims which are not released as set forth above, that it will comply with all the provisions of the said grant including without limitation those provisions relating to notification to the Grant Officer and relating to the defense or prosecution of litigation.

IN WITNESS WHEREOF, this assignment has been executed this 4TH day of August, 2014.

Ed Blaustein
(Grantee)
Ed Blaustein
Director of Finance

CERTIFICATE

I, William E. Slade, certify that I am the Secretary (Official Title) of the corporation named as Grantee in the foregoing release; that Ed Blaustein, who signed said release on behalf of the Grantee was then Director of Finance (Official Title) of said corporation; that said release was duly signed for and in behalf of said corporation by authority of its governing body and is within the scope of its corporate powers.

W.E. Slade

(CORPORATE SEAL)

REQUEST FOR ADVANCE OR REIMBURSEMENT		OMB APPROVAL NO. 0348-0004		PAGE 1 OF PAGES 1
X USUHS/TSNRP Reimb. Direct Fund Cite		1 TYPE OF PAYMENT REQUESTED	a. "x" one or both: Advance / X Reimbursement; b. "x" the applicable box: Final / X Partial	2. BASIS OF REQUEST: CASH / X ACCRUAL
3. FEDERAL SPONSORING AGENCY AND ORGANIZATION ELEMENT TO WHICH THIS REPORT IS SUBMITTED Certifying Office: USUHS Vendor Pay Station: DFAS Indianapolis 8899 E. 56th Street Indianapolis, IN 46249-3800		4. FEDERAL GRANT OR OTHER IDENTIFYING NUMBER ASSIGNED BY FEDERAL AGENCY HT9404-12-1-TS13 USUHS Project # N12-P12		5. PARTIAL PAYMENT REQUEST NUMBER FOR THIS REQUEST 26-34088
6. EMPLOYER IDENTIFICATION NUMBER	7. RECIPIENT'S ACCOUNT NUMBER OR IDENTIFYING NUMBER	8 PERIOD COVERED BY THIS REQUEST FROM (month, day, year) 09/01/13 Anticipated Period		TO (month, day, year) 10/31/13
52-1317896	306141-1.00-63891			
9. RECIPIENT ORGANIZATION The Henry M. Jackson Foundation for the Advancement of Military Medicine 6720 Rockledge Drive, Suite 100 Bethesda, MD 20817		10. PAYEE (Where check is to be sent is different than item 9) Name Same as #9 Number and Street		

11 COMPUTATION OF AMOUNT OF REIMBURSEMENTS/ADVANCES REQUESTED

PROGRAMS/FUNCTIONS/ACTIVITIES		[A]	[B]	[C]		TOTAL
a. Total program outlays to date						52,473.18
b. Less Cumulative program income						0.00
c. Net program outlays (Line a minus b)						52,473.18
d. Estimated net cash outlays for advance period						-
e. Total (Sum of lines c& d)						52,473.18
f. Non-Federal share of amount on line e						0.00
g. Federal share of amount on line e						52,473.18
h. Federal payments previously requested						52,179.28
i. Federal share now requested (Line g minus h)					Total Amount Due This Invoice	293.90
j. Advances required by month, when requested by Federal grantor agency for use in making prescheduled advances	1st month					
	2nd month					
	3rd month					

12 ALTERNATE COMPUTATION FOR ADVANCES ONLY

a. Estimated Federal cash outlays that will be made during period covered by the advance	
b. Less Estimated balance of Federal cash on hand as of beginning of advance period	
c. Amount requested (Line a minus line b)	

13 CERTIFICATION

I certify that to the best of my knowledge and belief the data on the reverse are correct and that all outlays were made in accordance with the grant condition other agreement and that payment is due and has not been previously requested.	SIGNATURE OF AUTHORIZED CERTIFYING OFFICIAL	DATE REQUEST SUBMITTED
Laraine Peng, Billing Manager Telephone (240) 694-2008 — Date 11/25/13	TYPED OR PRINTED NAME AND TITLE	TELEPHONE (AREA CODE, NUMBER, EXT.)

This space for agency use

97 20130 1885 HL3D 9762 302701 410051 044322 293.90

USUHS TRACKING NUMBER: 014

AUTHORIZED FOR LOCAL REPRODUCTION

Standard Form 270 (Rev. 2-92)
Prescribed by Office of Management and Budget
Cir No. A-102 and A-110

Remittance Information :::

ENT Number:	Rem Type:	Remit Number:	Remit Date:	Date Qualifier:	Original Amount:	Discount Amount:	Amount Paid:
1	IV	26-34087	12/10/2013	003	$8,661.12		$8,661.12

Reference Identification

Reference Identification Qualifier: GC — Reference Identification: HT9404121TS12
Reference Identification Qualifier: IL — Reference Identification: 905794
Reference Identification Qualifier: VV — Reference Identification: 9965200
Reference Identification Qualifier: CK — Reference Identification: 041036009500043

Remittance Information :::

ENT Number:	Rem Type:	Remit Number:	Remit Date:	Date Qualifier:	Original Amount:	Discount Amount:	Amount Paid:
1	IV	26-34088	12/10/2013	003	$293.90		$293.90

Reference Identification

Reference Identification Qualifier: GC — Reference Identification: HT9404121TS13
Reference Identification Qualifier: IL — Reference Identification: 905794
Reference Identification Qualifier: VV — Reference Identification: 9965200
Reference Identification Qualifier: CK — Reference Identification: 041036009500043

6389 / Oct-13

Remittance Information :::

ENT Number:	Rem Type:	Remit Number:	Remit Date:	Date Qualifier:	Original Amount:	Discount Amount:	Amount Paid:
1	IV	26-34363	01/06/2014	003	$494,630.23		$494,630.23

Reference Identification

Reference Identification Qualifier: GC — Reference Identification: HU00011020002
Reference Identification Qualifier: IL — Reference Identification: 905794
Reference Identification Qualifier: VV — Reference Identification: 9965200
Reference Identification Qualifier: CK — Reference Identification: 041036009500043

Remittance Information :::

ENT Number:	Rem Type:	Remit Number:	Remit Date:	Date Qualifier:	Original Amount:	Discount Amount:	Amount Paid:
1	IV	26-34383	01/06/2014	003	$106,935.52		$106,935.52

Reference Identification +6358

Reference Identification Qualifier: GC — Reference Identification: HU00011110004
Reference Identification Qualifier: IL — Reference Identification: 905794
Reference Identification Qualifier: VV — Reference Identification: 9965200
Reference Identification Qualifier: CK — Reference Identification: 041036009500043

Remittance Information :::

ENT	Rem	Remit Number:	Remit Date:	Date Qualifier:	Original	Discount	Amount Paid:

Payment/Remittance Detail :::

Originator : DFAS DA/USUHS ERP
Originator ID : 3041036004
Receiver : HENRY M JACKSON FOUNDATION FOR

Bank Originating Transaction
Routing ID: 041036004
Account: 8522

Bank Receiving Transaction
Routing ID: 051000017
Account: 8403496

43)59

Reference Number :	Payment Date :	Payment Method:	Standard Entry Class:	C/D Flag:	Currency:	Amount :
041036009500043	02/13/2014	ACH	CTX	C		$3,387,211.16

Notes/Special Instructions

Note Ref Code: CHG Description: CTX MISMATCH: BPR VAL REPLACED BY ACH VAL BELOW.
Note Ref Code: CHG Description: BPR13:052001633 ACH6/3&4:051000017

Trace

Trace Type Code:	Reference Identification:	Originating Company Identifier:	Reference Identification:
1	041036009500043		

$023 430 144 5716

Date/Time Reference

Date/Time Qualifier:	Date:	Time:
097	02/12/2014	12:00 AM

Name & Address Information :::

Entity Identifier Code: PE Name: HENRY M JACKSON FOUNDATION FOR Identification Code Qualifier: 93
Identification Code: 0HC11

Name & Address Information :::

Entity Identifier Code: PR Name: DFAS DA/USUHS ERP Identification Code Qualifier: 10
Identification Code: 00008522

Entity Summary Information :::

Assigned Number:	Ref ID Qualifier:	Ref ID:	Entity ID Code:	ID Code Qualifier:	ID Code:
1					

Remittance Information :::

ENT Number:	Rem Type:	Remit Number:	Remit Date:	Date Qualifier:	Original Amount:	Discount Amount:	Amount Paid:
1	IV	08-34373	01/06/2014	003	$290,442.09		$290,442.09

Reference Identification

Reference Identification Qualifier: GC Reference Identification: HU00010220005
Reference Identification Qualifier: IL Reference Identification: 905794
Reference Identification Qualifier: VV Reference Identification: 9965200
Reference Identification Qualifier: CK Reference Identification: 041036009500043